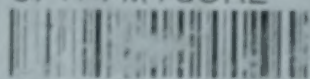


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Fungi, Bacteria,

Viruses, microorganisms,

Culture media, Disinfectants,

Pathogens, Immunity,

Rickettsial diseases.

TLMS





# MICROBIOLOGY

by

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Microbiology..

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Affectionately  
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to  
E. F. K.  
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D. B. H.

*"...Modern science is physics, while medieval science was something at once less potent and more important, ethics. With ethics alone, man may love the good, but never find it; with physics alone he may gain the whole world, and lose his own soul."*

*—Randall: Making of the Modern Mind,  
Houghton Mifflin Company.*



## PREFACE

This textbook is intended for the student who will require a comprehensive knowledge in the fields of general and pathogenic microbiology. An effort has been made to present currently accepted facts and theories simply and at the same time to include enough information to permit an adequate understanding of the subject and to be of practical value. The text divides naturally, into two parts, progressing from a general discussion of the microscopic world to pathogenic microorganisms and the diseases which they produce. The first part emphasizes the large phylogenetic groups of microorganisms, their biology and interrelationships, bacterial physiology, microbial populations and their control, as well as methods for the study of microorganisms; the second part treats of parasitism and disease, immunity and the pathogenic bacteria, viruses, rickettsiae, fungi and protozoa. References to summarizing articles and to books for more detailed reading are included at the back of the book and should be of value to the enterprising student.

The authors wish to express their appreciation to their friends and colleagues of both The University of Oklahoma School of Medicine and The University of Chicago who have helped in so many ways during the preparation of this book. We are especially indebted to Dr. G. M. Dack and Dr. Stewart A. Koser for reading and criticizing the manuscript and to Dr. George Gomori, Dr. Eleanor M. Humphreys, Dr. Karl Pribram and Dr. Kirsten Vennesland for many illustrations. Many of the drawings and diagrams were painstakingly executed by Mr. Ernest F. Hiser, and the Committee on Materials for Visual Instruction in Microbiology of the Society of American Bacteriologists also supplied certain illustrative materials. We are particularly grateful to Dr. Mark R. Everett, Dr. Hiram D. Moor, Dr. Homer F. Marsh, Dr. Donald B. McMullen, and Dr. Lucy G. Taliaferro for their advice and encouragement.

FLORENE C. KELLY  
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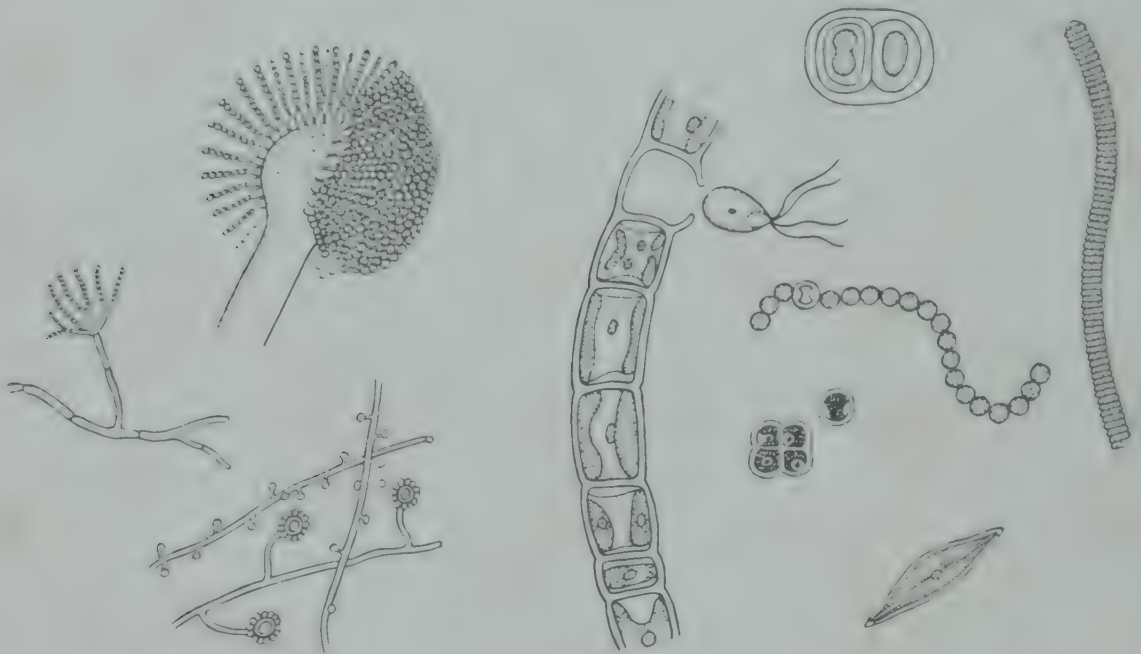
INVISIBLE

RICKETTSIAE

VIRUSES

BACTERIA

YEASTS



MOLDS

ALGAE



PROTOZOA

Fig. 7. Various types of microorganisms. (Magnification approximately  $\times 300$ )

## THE NATURE OF LIVING ORGANISMS

**Protoplasm, the Physical Basis of Life.** In order to recognize the various kinds of microorganisms one must know the properties common to all living organisms and the characteristics which distinguish plants from animals. All plants and animals are made of protoplasm and to understand the essential character of living organisms it is necessary to be familiar with the nature of this living stuff. For this purpose a review of certain fundamental facts of biology and chemistry is in order.

One might suppose that the vital manifestations of protoplasm are due to unique elements not found in the nonliving world. Analysis of protoplasm, however, shows that it is composed of familiar elements, chiefly carbon, oxygen, hydrogen and nitrogen, with smaller amounts of potassium, phosphorus, sulfur, calcium, magnesium, sodium, iron, chlorine and traces of other elements. Living cells are 70 to 90 per cent water. In nature, only living protoplasm can combine these ordinary elements into carbohydrates, fats and proteins. Carbohydrates are compounds of carbon, hydrogen and oxygen ranging from simple sugars (monosaccharides) such as glucose ( $C_6H_{12}O_6$ ) to complex polysaccharides, like cellulose, glycogen and starch, which are polymers or multiples of the monosaccharides. The fatty compounds contain the same elements as the carbohydrates, but in different proportions and arrangements. Proteins are the largest and most complicated of the organic compounds in protoplasm. They are composed of nitrogen as well as carbon, hydrogen and oxygen; the majority contain sulfur and a few have phosphorus or iron in the molecule. Each molecule is an aggregate of several to many amino acids which are said to be the "building blocks" of the proteins. Each amino acid is an organic acid in which at least one hydrogen atom has been replaced by an amino group ( $NH_2$ ). There are known to be over twenty amino acids which may be combined in many ways to form a great variety of protein molecules. In addition to these organic compounds, protoplasm contains small quantities of a number of different mineral salts. The protoplasm of all organisms has the same general composition (it is a mixture of carbohydrates, fats, proteins, mineral salts and water), but the chemistry of no two species and probably of no two individuals is exactly alike. Differences between organisms are due to inherent dissimilarities in their protoplasm.

Naked protoplasm as seen in the simplest of one-celled animals, the amoeba, is a translucent, colorless to grayish, slimy substance of variable viscosity. Embedded in this ground substance are many granules, globules and vacuoles. Protoplasm can exist as a free flowing fluid or a more rigid jelly, and its consistency may vary not only in different kinds of cells but also from time to time in the same cell. The proteins responsible for this and many other properties of protoplasm are highly sensitive to chemical and physical changes in the environment. Due to its proteins and fats, protoplasm is a colloid, *i.e.*, it is a substance in which finely divided particles, which are too large to go into true



solution and too small to settle out, are permanently dispersed in a liquid medium.

A great deal is known about the individual mechanisms by which the functions of protoplasm are performed, but in the last analysis the nature of the life-giving property with which protoplasm is endowed is still unknown. It has been said of protoplasm that the sum is greater than its parts. It is due to the activity of protoplasm that the organism is alive, that it grows, reproduces and responds to changes in the environment.

*Organization of Protoplasm.* Protoplasm occurs in units known as cells. Large organisms are made of billions of these microscopic units, while microscopic organisms may be composed of just one to many cells. Individual cells may be capable of independently carrying on all the functions of life (these are unicellular organisms) whereas others are dependent in part upon the activities of neighboring cells as they are in the multicellular organism. The protoplasm of both one-celled and multicellular plants and animals is usually organized within each cell according to a fairly definite plan. Thus the protoplasm of the typical cell is differentiated into the cytoplasm and the nucleus. Cytoplasm makes up the bulk of the cell and the nucleus is generally a centrally placed body embedded in the cytoplasm. At the periphery of the cell the protoplasm is separated from the environment by a limiting plasma membrane which is itself a thin layer of modified cytoplasm. The typical plant cell is enclosed in a rigid cell wall of cellulose, a nonliving material laid down over the plasma membrane. Animal cells do not have such a cell wall, and this freedom from a stiff covering round each protoplasmic unit allows the animal body more flexibility than is found in plants.

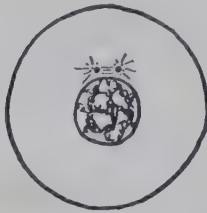
The ground substance of cytoplasm has the physical properties already described for protoplasm. Embedded in it are inclusions such as granules of reserve food, vacuoles filled with droplets of fat or water, and crystals as well as certain formed elements including the Golgi bodies and mitochondria, centrioles in animal cells, and in plant cells chlorophyll-containing bodies named chloroplasts.

In stained preparations the nucleus usually appears as a round to oval, dark, granular body. In its deep-staining granules of **chromatin** are located the **genes** or factors which determine the inheritance of the organism. A mesh of light-staining material, the **linin network**, supports the chromatin granules, and it is these structures which become transformed into the **chromosomes** when the cell prepares itself for division. At cell division each chromosome is split in half longitudinally by a complicated process known as **mitosis**. In this way equal qualitative and quantitative distribution of the chromosomes to the two new daughter cells is assured. In addition to bearing the hereditary factors the nucleus plays a role in controlling the use of foods by the cell as well as all growth and reproduction. More than one nucleus may be present in certain cells.

## MITOSIS IN AN ANIMAL CELL



Resting stage



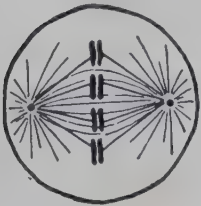
Early prophase



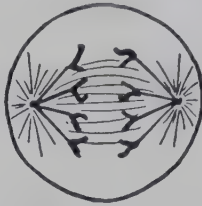
Prophase



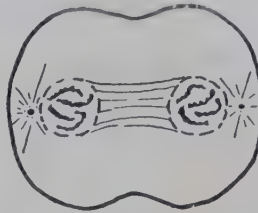
Late prophase



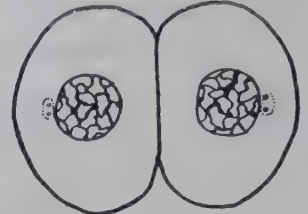
Metaphase



Anaphase



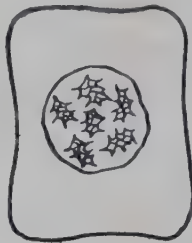
Telophase

Resting stage  
with two nuclei

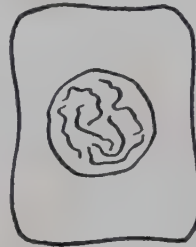
## MITOSIS IN A PLANT CELL



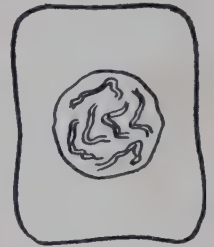
Resting stage



Early prophase



Prophase



Late prophase



Metaphase



Anaphase



Telophase

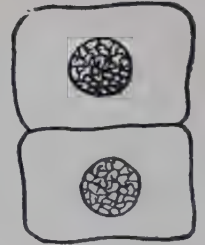
Resting stage with  
two nuclei

Fig. 8. Diagrams of mitosis in an animal cell and in a plant cell. (From Mavor: *General Biology*, The Macmillan Co.)

**Life Processes.** The activities necessary to maintain life are fundamentally the same in all organisms; they are performed by a single cell in the unicellular organism, and by a variety of tissues cooperating in the higher, multicellular individual. The characteristics of living organisms are the result of these protoplasmic activities: **metabolism**, which allows for **maintenance**, **growth** and **reproduction** of the organism, and **irritability**, which results in **coordination** of the individual and **adaptation** to its environment.

**Metabolism.** In nature only living organisms can capture the potential energy bound up in foods and transform it into the kinetic energy necessary



for work and the maintenance of life. Just remaining alive involves work, for protoplasm is continuously wearing out. The cellular carbohydrates, fats and proteins are forever being destroyed and rebuilt during life. The organism's chief activity, involving great expenditure of energy, is therefore the constant repair of the protoplasm. The chemical changes by which an organism breaks down and rebuilds its protoplasm are known collectively as the metabolism of that organism. The chemical reactions which lead to the break-down of protoplasmic constituents and the release of energy are summed up in the term **catabolism**, and the constructive processes resulting in the building up or synthesis of protoplasm are termed **anabolism**. To maintain life the anabolic rate must keep pace with that of the catabolic activities.

Organisms must sustain an exchange of substances between their cells and the environment. Foods (or, in the case of green plants, substances that can be made into foods) and oxygen are taken in and the waste products of catabolism are eliminated into the surroundings. An organism's food must supply substances which can be used to replace its worn out protoplasm and to provide energy. For proper nutrition animals and most fungi require the carbohydrates, fats and proteins of other animal and plant bodies as well as mineral salts and water. Green plants, on the other hand, can manufacture the complex organic compounds of their protoplasm from simple inorganic materials due to the power of their chlorophyll to obtain energy directly from sunlight. This **photosynthesis** of green plants will be more thoroughly discussed elsewhere. It is enough to note here that the ultimate source of energy for life on the earth is the sun, and that animals and most colorless plants are directly or indirectly dependent on the green plants for food.

Oxidation of foods in the cells results in the liberation of energy from these substances which are ultimately decomposed to the waste products carbon dioxide and water. In larger animals the intake of oxygen and the release of carbon dioxide and water vapor from the body are accompanied by visible breathing movements, and to those unfamiliar with cellular metabolism breathing is synonymous with respiration. However, **respiration** is more than a superficial exchange of gases; it is the life process by which every cell secures energy through the oxidation of substances within the cell. Regardless of how they obtain their foods, all organisms have this in common: they must continue to utilize energy. When the energy-transforming system which is protoplasm ceases to function, the organism dies; its body decomposes and returns to the nonliving world.

The constant break-down of cellular proteins yields nitrogenous wastes and results in processes of **excretion**. Due to differences in nutrition and activity, animals accumulate more nitrogenous excretions than plants, and special excretory structures have been developed in unicellular and multicellular animals to collect and discharge from the body these liquid wastes containing such products as urea, uric acid and ammonia.

**Growth and Reproduction.** In young organisms anabolism exceeds catabolism, *i.e.*, the synthesis of protoplasm goes on at a greater rate than its breakdown, and growth results. Growth in itself is not peculiar to living organisms (icicles and crystals grow), but only living cells can grow by transforming foods into more of their own substance, protoplasm. Another outstanding property of living things is their power to produce more of their own kind. Every plant and animal inherits a size limit. It grows so big and then stops. When the limit of growth and functional maturity has been attained, the organism is said to be an adult. In the adult anabolic and catabolic activities are balanced, and growth of the individual ceases, but the adult is capable of reproducing one or more new individuals like itself. Reproduction involves cell division and the synthesis of more protoplasm; reproduction is actually a continuation of growth, or, in a sense, it is an over-growth of the adult. This idea is nicely illustrated by unicellular organisms that grow to their size limit and then divide to form two new individuals each of which must synthesize enough protoplasm to reach the status of an adult before it too divides. The progressive changes through which an organism passes from youth through adulthood and reproduction to a new, young individual similar to the one from which it originated constitutes the **life cycle** of the organism.

**Irritability, Coordination and Adaptation.** Protoplasm and, consequently, all living organisms are **irritable**, *i.e.*, they respond to changes in the surroundings. A change in the environment which elicits a response in the organism is known as a **stimulus**. Organisms exhibit different responses to physical stimuli, such as changes in temperature, light and pressures, as well as to chemical stimuli. Motion in any direction and cessation of movement are responses to stimulation. If cells have the power to elaborate and release substances into their surroundings, this act of **secretion** or its inhibition is in response to a stimulus. Not only does the part of the organism in contact with the stimulus respond, but the whole organism behaves as a coordinated unit. This coordination is possible because the protoplasm **conducts** the stimulus as an impulse to all parts of the organism. The intake of food and oxygen, the subsequent metabolic changes and, in fact, the very life of the organism are, in final analysis, the result of interplay between the organism and its environment, each act or reaction being a response to a stimulus. Thus the organism adapts itself to its surroundings, and as long as it is able to fit itself into harmonious relations with the ever-changing conditions about it, life continues.

**Enzymes.** The living cell carries on its functions largely by means of substances known as enzymes. An **enzyme** may be inadequately but simply defined as an **organic catalyst** produced by a living organism. A catalyst may be regarded as a substance which alters the speed of a chemical reaction in which the catalyst itself participates but is not permanently changed. A familiar example of an inorganic catalyst is spongy platinum that stimulates the combination of gaseous hydrogen and oxygen to form water, but itself is not used



up in the reaction. Another instance of catalysis produced by an inorganic agent is the accelerating effect of manganese dioxide on the decomposition of potassium chlorate. The latter substance may be melted at a certain temperature with the evolution of very little oxygen. If, however, a pinch of powdered manganese dioxide is added under the same conditions, an abundance of oxygen is liberated and at the end of the reaction the manganese dioxide may be recovered unchanged. The nature of enzymes, or the catalysts produced by living cells, is not entirely understood, but they are usually regarded as being complex protein molecules or as active groups attached to certain protein molecules. In general, one enzyme will catalyze only one or a few chemically similar reactions and will be inactive in other reactions. This **specificity** of action is an outstanding property of enzymes. The physical and chemical nature of the surroundings greatly influence enzymatic action, and enzymes are readily destroyed by certain environmental changes. They are sensitive to variations in temperature, hydrogen ion concentration ( $pH$ ), oxygen tension, light, concentration of inorganic salts and moisture. Their action may be inhibited by certain dyes and other chemicals acting as enzyme "poisons," or it may be accelerated by the presence of certain substances which serve as enzyme activators.

The nature of microorganisms as well as that of larger organisms reflects the character of their enzymes. Thus a species of bacteria is said to have a certain optimum temperature, to be aerobic or anaerobic, or to need a neutral solution in which to grow. These requirements are determined by the temperature, oxygen and  $pH$  relations of the enzymes of that species. The substances that can serve an organism as food are determined largely by the kinds of enzymes possessed by that organism.

**Enzyme Systems.** Tissue cells and unicellular organisms must possess a variety of enzymes, and the wider the variety the greater the adaptability of the organism to its environment. Organisms are said to produce whole batteries of enzymes to act upon a variety of substances. As knowledge of the biochemistry of microorganisms increases it is evident that many more enzymes are involved than was once supposed. What was formerly thought to be a single chemical reaction catalyzed by one enzyme has often proved to be a series of reactions in which several enzymes take part, each enzyme reacting with a product of the previous chemical change. Hence it is now common to speak of an **enzyme system** participating in a series of step-wise reactions. For example, the production of alcohol from sugar was once said to be catalyzed by the yeast enzyme **zymase**. We now know that the alcoholic fermentation of sugar involves several chemical reactions catalyzed by as many different enzymes. In other words, the zymase of yeast is not a single substance but a complex enzyme system.

**Nomenclature and Classification.** Although enzymes are always formed by living cells, their action may be independent of the cell's presence. Many enzymes leave the cell in which they originate and operate in the surrounding medium. More is known about these extracellular or **exoenzymes** than about



most of those which act within the cell, the intracellular or **endoenzymes**. Important among the **exoenzymes** are those which prepare foods for absorption and utilization by the cells such as the digestive enzymes of animals and the fungi.

In modern practice enzymes are usually classified and named according to (1) the kind of substrate upon which they act or (2) the kind of chemical reaction they produce. In each case the suffix **-ase** is added to an appropriate stem to form the name of the enzyme. Thus according to the first scheme three important classes of enzymes are **proteinases**, **lipases** and **carbohydrases** which decompose proteins, fats and carbohydrates respectively. Since the decomposition of these foods involves a dissolving or **lytic** action, those attacking proteins are also known as **proteolytic** enzymes, the fat-digesting enzymes are described as **lipolytic**, while those acting on starch and sugars are termed **amylolytic** and **saccharolytic** respectively. Individual enzymes included in these general classes are named after the specific substances they attack. These are designated by such names as **gelatinase**, **urease**, **pectinase**, **sucrase**, **lactase** and **maltase**. The second method of naming enzymes is based on the type of chemical action catalyzed; for example, certain general classes of enzymes are known as **oxidases**, **dehydrogenases**, **hydrolases**, etc.

The older nomenclature which developed primarily from the study of animal physiology follows no definite plan. For instance, the starch-digesting enzyme of the saliva is named **ptyalin**. **Pepsin** and **trypsin** are proteolytic enzymes of the animal's digestive tract and **rennin** is a milk-coagulating enzyme produced in the stomach of mammals as well as by some microorganisms.

**Identification of Enzymes.** Relatively little is known of the chemical nature of microbial enzymes, by their presence may be determined by the chemical changes they produce. Given a certain substrate and standard conditions, a particular enzyme will always act upon this substrate in a definite way to form certain products. Since the chemistry of a living organism (biochemistry) depends on the enzymes it produces and since each species inherits a given set of these specific enzymes, it follows that a study of the biochemistry of an unknown microorganism and the identification of its enzymes will contribute materially to the identification of the microorganism itself.

## CLASSIFICATION AND SCIENTIFIC NAMES OF ORGANISMS

The living world is divided into two great kingdoms, the Animal Kingdom and the Plant Kingdom. Closely related animals share certain characteristics not found in other animals, and the same is true of plants. These distinguishing and relating characteristics form the bases for classification of the Animal Kingdom into main subgroups or **Phyla** and of the Plant Kingdom into **Divisions**. Each **Phylum** or **Division** is in turn broken down into smaller and smaller subgroups of organisms more and more closely related until in the small-

est subgroup only minor variations exist between individuals. The plan for the classification of living organisms includes these subgroups:

Kingdom  
Phylum (Division)  
Class  
Order  
Family  
Tribe  
Genus  
Species

Minor variations are always present in a species, and no two individuals are exactly alike. If some members of a species share one or more of these variations they may be considered a **subspecies**, **type**, **variety** or **strain** of that species. The student should remember that taxonomy, the science of classification, is an arbitrary scheme to satisfy man's desire for a system to combine order with knowledge. The scheme is not perfect. Authorities do not always agree as to whether or not a certain variety of organism should be considered a new species. Intermediate forms, such as *Euglena*, have been classified in both the animal and plant kingdoms. Classifications may be changed, usually either by the discovery of new organisms or the description of new relationships between living things. The renaming and rearrangement of bacteria into new subgroups are not uncommon.

In practice an organism is named according to the genus and species to which it belongs, *i.e.*, according to the system of **binomial nomenclature** (the two-name system). Thus man has the scientific name *Homo sapiens*; man belongs to the genus *Homo* and species *sapiens*. The scientific name of a protozoan belonging to the genus *Paramecium* and the species *caudatum* is *Paramecium caudatum*. The name of the genus is capitalized while the species name is begun with a small letter. Scientific names of organisms are commonly designated by underscoring or italics.

TABLE 1. CLASSIFICATION OF THE PLANT KINGDOM

Division <i>Thallophyta</i> .	Undifferentiated plants having no true roots, stems or leaves.
Subdivision <i>Algae</i> .	Thallophytes possessing chlorophyll. (Includes microscopic green plants and the sea weeds.)
Subdivision <i>Fungi</i> .	Thallophytes possessing no chlorophyll. (Includes mushrooms, toadstools and other fleshy fungi as well as molds, yeasts and bacteria.)
Division <i>Bryophyta</i> .	Mosses and liverworts.
Division <i>Pteridophyta</i> .	Ferns, horsetails and ground pines.
Division <i>Spermatophyta</i>	Seed plants, cone-bearing evergreens and flowering plants.

TABLE 2. CLASSIFICATION OF THE ANIMAL KINGDOM

Phylum <i>Protozoa</i> .	One-celled animals.
Phylum <i>Porifera</i> .	Sponges.
Phylum <i>Coelenterata</i> .	Hydra; jellyfish; sea anemones; corals.
Phylum <i>Platyhelminthes</i> .	Flatworms: <i>Planaria</i> , flukes, tapeworms.
Phylum <i>Nemathelminthes</i> .	Round worms: vinegar "eel"; hookworm; <i>Trichinella</i> ; <i>Ascaris</i> .
Phylum <i>Rotifera</i> .	Rotifers, microscopic, fresh-water "wheel animal-icules."
Phylum <i>Bryozoa</i> .	"Moss animals" in colonies encrusting plants, rocks and animal shells.
Phylum <i>Brachiopoda</i> .	Bivalved "lamp shell" animals.
Phylum <i>Echinodermata</i> .	Spiny-skinned animals: starfishes; brittle stars; sea urchins; sea cucumbers; sea lilies.
Phylum <i>Mollusca</i> .	Chitons; snails; slugs; squid; octopus; oyster; clam.
Phylum <i>Annelida</i> .	Segmented worms: clam worm; earthworm; leeches.
Phylum <i>Arthropoda</i> .	Joint-footed animals: crabs; lobsters; centipedes; millipedes; insects; scorpions; spiders; ticks; mites.
Phylum <i>Chordata</i> .	Animals with a "back string" or notochord.
Subphylum <i>Hemichorda</i> .	<i>Balanoglossus</i> .
Subphylum <i>Urochorda</i> .	Sea squirts.
Subphylum <i>Cephalochorda</i> .	<i>Amphioxus</i> .
Subphylum <i>Vertebrata</i> .	Animals with a vertebral column or backbone.
Class <i>Cyclostomata</i> .	Lamprey; hag fishes.
Class <i>Elasmobranchii</i> .	Shark; dogfish; ray; skate.
Class <i>Pisces</i> .	True or bony fish.
Class <i>Amphibia</i> .	Salamander; frog; toad.
Class <i>Reptilia</i> .	Turtle; snake; alligator; crocodile.
Class <i>Avis</i> .	Birds.
Class <i>Mammalia</i> .	Kangaroo; opossum; bat; sea lion; armadillo; whale; man.



# 3

## THE PROTOZOA

**Distinguishing Characteristics.** All one-celled animals belong to the phylum Protozoa, the first or lowest subdivision of the animal kingdom. While a few of the largest are barely visible to the unaided eye, most protozoa are microscopic. Their cellular organization ranges from that of the amoeba, a bit of naked protoplasm, to the complicated organization of the ciliates, organisms possessing highly specialized structures or **organelles** for coordination, motility, the capture of food and protection. The single cell which constitutes each organism is differentiated into cytoplasm and nucleoplasm. Generally there is a distinct demarcation between a clear layer of outer cytoplasm or **ectoplasm** and the more granular, inner mass of cytoplasm, the **endoplasm**. Food vacuoles, one or more contractile vacuoles, one or more nuclei and other structures concerned with the cell's activities are usually embedded in the granular endoplasm. The typical protozoan possesses a mouth or **cytostome** and a thin flexible cell membrane or **pellicle**. Chlorophyll is not present. These are strictly animal characteristics. Their manner of moving, feeding and ridding their bodies of waste materials also mark them as animals.

Exceptions to this typical pattern occur; for example, the amoebae have no limiting pellicle and therefore no well defined form, and certain of the flagellates contain chlorophyll. Many protozoa which live in association with higher organisms, *i.e.*, parasites, lack structures which in their free-living relatives are associated with the intake of solid food. Despite such deviations from the type and with the exception of the green flagellates, the structure and behavior of these unicellular organisms is predominantly animal, and they are not easily mistaken for algae or fungi.

The majority of protozoa exist as solitary individuals, but in the so-called **colonial protozoa** a number of individuals are associated in a **colony**. These organisms are usually held together by connecting strands of cytoplasm, by an intercellular matrix of jelly-like material or both. As distinguished from the interdependent cells of multicellular animals, each protozoan cell in a colony is an independent organism performing the life processes necessary to the individual. In certain colonies, however, reproduction may be limited to specialized individuals, and then the colony is said to be composed of reproductive and vegetative or somatic cells.

**Classes of Protozoa.** The phylum Protozoa is divided into four classes mainly on the basis of method of locomotion. In these classes there are about 20,000 different species, the majority of which are free-living. The rest are parasites and a few of these may cause important diseases in man and animals. In the following outline the chief and distinguishing characteristics of the four classes of protozoa are given. Where two examples of a class are cited the first is a free-living and the second a parasitic protozoan.

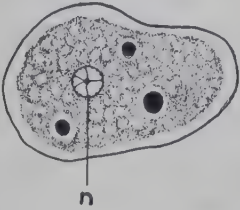
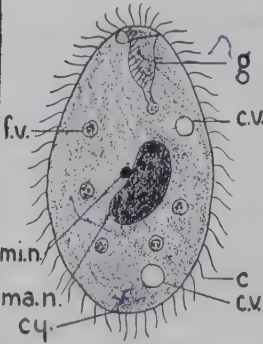
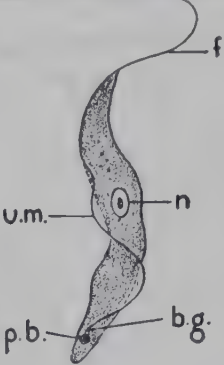
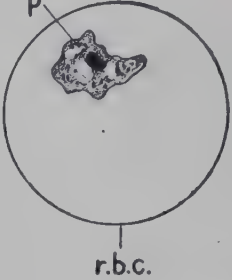
SARCODINA	INFUSORIA	MASTIGOPHORA	SPOROZOA
 x 1000	 x 500	 x 1500	 x 3000
ENDAMOEBA HISTOLYTICA	BALANTIDIUM COLI	TRYPANOSOMA GAMBIENSE	PLASMODIUM VIVAX

Fig. 9. Classes of Protozoa. b.g, Basal granules; c, cilia; c.v, contractile vacuole; cy., cytopyge or anal spot; f, flagellum; f.v, food vacuole; g, gullet; ma.n., macronucleus; mi.n., micronucleus; n, nucleus; p, *Plasmodium*; p.b., parabasal body; r.b.c., red blood cell; u.m., undulating membrane. (From Belding: *Textbook of Clinical Parasitology*, D. Appleton-Century Co., Inc.)

- ✓

Class SARCODINA.

Protozoa which move by extending temporary projections of flowing protoplasm known as **pseudopodia** (sing., **pseudopodium**, false foot).

Examples: *Amoeba proteus*  
*Endamoeba histolytica*
- 3

Class MASTIGOPHORA.

Sometimes named Class FLAGELLATA. Protozoa which move by the lashing of one or more permanent, long, whip-like processes or **flagella** (sing., **flagellum**, a whip).

Examples: *Euglena viridis*  
*Trypanosoma gambiense*
- ✓

Class INFUSORIA.

Sometimes designated Class CILIATA. Protozoa which move by the coordinated beating of **cilia** (sing., **cilium**, eyelash), fixed protoplasmic projections differing from flagella in that they are finer, shorter, and more numerous.

Examples: *Paramecium caudatum*  
*Balantidium coli*



## 4 Class SPOROZOA

Possess no locomotor organelles. All obligate parasites. Typical life cycle includes asexual reproduction by multiple fission (schizogony) often in a vertebrate host, and sexual reproduction resulting in the production of sporozoites (sporogony) usually in an insect.

Example: *Plasmodium malariae*

**Habitat.** The free-living protozoa are widely distributed in the soil and in bodies of salt and fresh water. Almost any sample of natural water taken from a spring, brook, river, or swamp will become a teeming menagerie of protozoa if a small quantity of organic matter is added and the sample is allowed to stand. Pieces of hay, kernels of wheat or other grains supply food and in some cases more protozoa to the water. An infusion of fresh horse manure will produce a spectacular culture of protozoa in a few days. In such an infusion the ciliates generally predominate at first, but in older cultures they may become scarce while the numbers of flagellates and amoebae increase. All protozoa are aquatic, i.e., each single cell must be bathed in water to grow. They may find conditions favorable for growth in ponds, oceans, or other natural bodies of water, in drops of moisture between soil particles, in the tissue fluids or intestinal contents of an animal body or in laboratory cultures.

Higher animals may be parasitized by several species of protozoa and man is no exception. Fortunately, most parasitic protozoa cause no harm to their hosts, but some are pathogenic, giving rise to such important diseases as malaria, amoebic dysentery and African sleeping sickness.

**Life Processes of Protozoa. Nutrition.** Like all animals, the protozoa differ fundamentally from plants in their nutrition. To obtain the complex organic foods they need, the free-living protozoa eat microscopic plants and animals smaller than themselves, i.e., bacteria, algae and other protozoa, or they may take in pieces of dead organisms. These solid foods and water are **ingested** through the mouth or in the amoebae are surrounded by the pseudopodia, and are **digested inside the body**. This typically animal mode of nutrition is termed **holozoic**. As the food and water pass into the cell proper they do not mix directly with the cytoplasm, but remain in a droplet which occupies a space in the cytoplasm, a **food vacuole**. New food vacuoles are formed as more food is ingested, and each is circulated around the cell by cytoplasmic movement. Digestive secretions are poured into the vacuole from the cytoplasm and by their action the solid foods are softened and transformed into soluble substances which are absorbed and utilized by the protoplasm. As absorption continues the food vacuole grows smaller and finally it is carried to the periphery of the cell. Any indigestible material taken in with the food is then eliminated or **egested** from the cell. As in all living organisms the digested foods supply materials and energy necessary for the synthesis of protoplasm and all other cellular activities. Parasitic protozoa may absorb the soluble food substances supplied by the host organism **directly through their cell membranes.**



## THE PROTOZOA

**Respiration.** Protozoa have no special structures for respiration. Oxygen dissolved in water diffuses into the cell through the cell membrane. In like manner carbon dioxide resulting from the oxidation of foods diffuses from the cell into the environment through the cell membrane. The contractile vacuoles which serve primarily in the function of excretion also eliminate carbon dioxide.

**Excretion.** With the exception of marine forms, protozoa are generally equipped with one or more special structures, **contractile vacuoles**, to rid the organisms of the waste products of metabolism and excess water. Soluble waste materials collect in these vacuoles which when filled contract and eliminate their contents through the cell's surface.

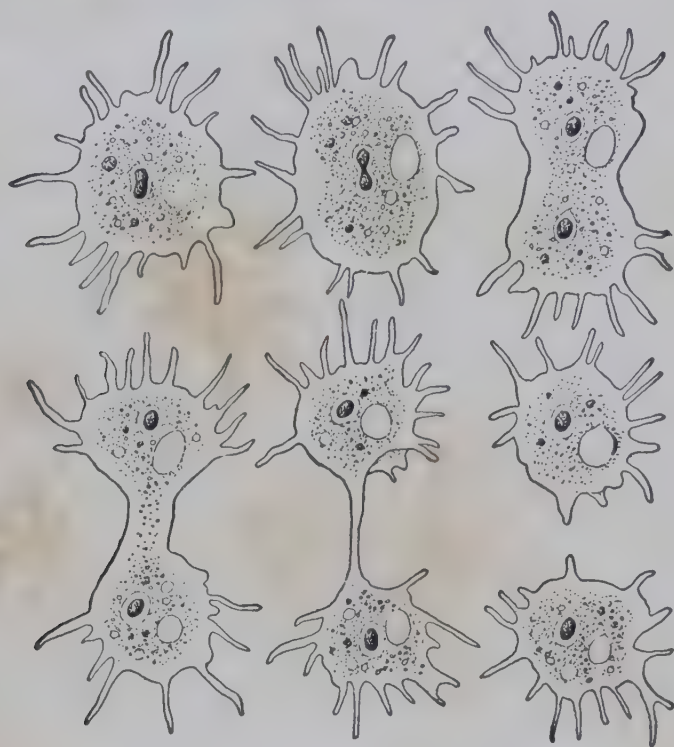


Fig. 10. An Amoeba in six successive stages of division. The dark body surrounded by a clear area is the nucleus. (Modified after Schultze, from Woodruff: *Foundations of Biology*, The Macmillan Co.)

**Reproduction.** Protozoa usually reproduce asexually by **binary fission**. A single nuclear division followed by equal division of the cytoplasm results in two equal-size daughter cells. In certain species, particularly of the Sporozoa, asexual reproduction is accomplished by **multiple fission** or **schizogony** by which a number of individuals are produced from a single parent cell. In this case the nucleus of the cell divides repeatedly or by a single fragmentation breaks up to form a number of nuclei in the mature cell. The cytoplasm then divides, producing as many uninucleate daughter cells as there were daughter nuclei. Less common among the protozoa is reproduction by **budding** or **unequal fission** in which one or more smaller individuals are produced by the parent organism.

After several asexual generations in some species, especially some of the ciliates, two individuals come together and exchange nuclear material during a temporary union termed **conjugation**. After conjugation the two organisms separate and each undergoes cell division. **Fertilization** or the permanent union of two cells occurs in most species of Sporozoa and less frequently among the Sarcodina and the green flagellates. The uniting cells (**gametes**) correspond to the sex cells of higher animals, and the single cell formed by their union is a **zygote**. Subsequent development and division of the zygote gives rise to a generation which reproduces asexually.

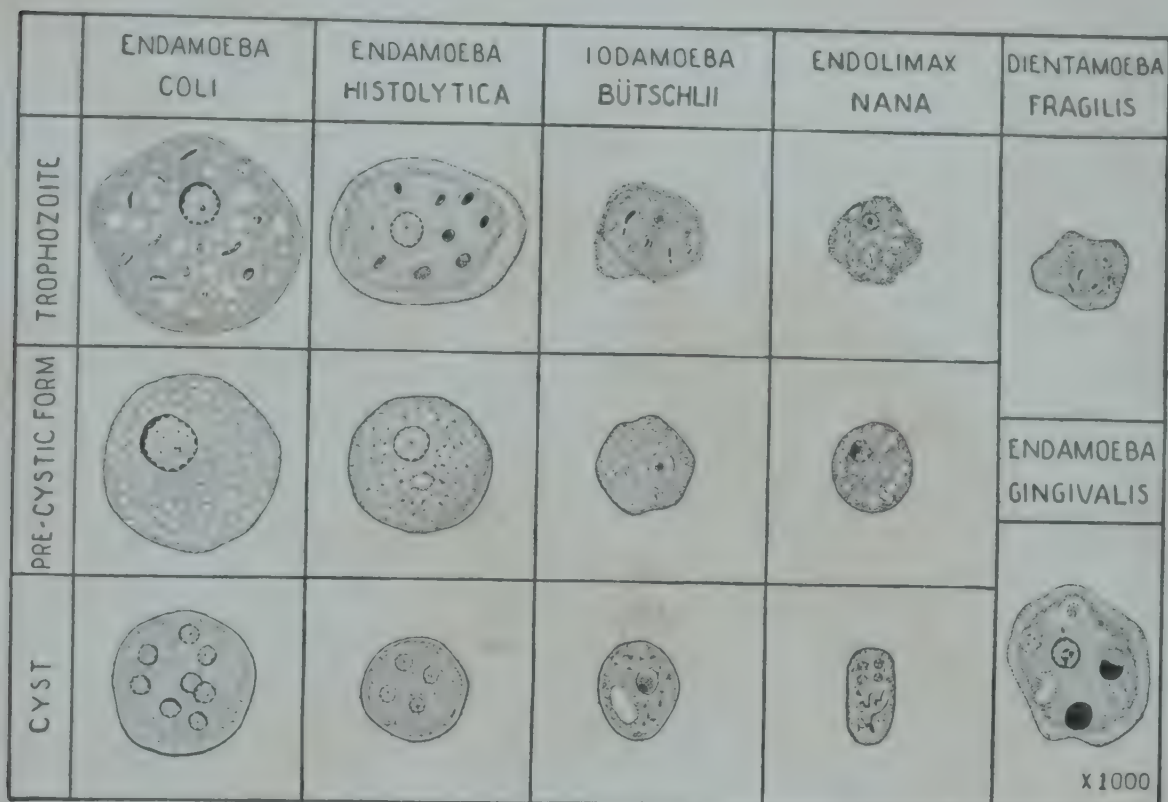


Fig. 11. Comparative morphology of amoebae which parasitize man. (From Belding: *Textbook of Clinical Parasitology*, D. Appleton-Century Co., Inc.)

**Encystment.** A factor which accounts for the widespread distribution of many free-living protozoa and for the survival of intestinal protozoa outside the body is their ability to form resistant **cysts**. In response to external stimuli or as a natural stage in their life cycle, these organisms may eliminate extraneous matter from their cells, assume a compact spherical or ovoid shape and secrete an enveloping wall of nonliving material. By this means they protect their protoplasm against drought, extremes in temperature, lack of food, injurious chemicals and other adverse conditions. In the cyst stage protozoa may lie dormant for long periods of time and may survive unfavorable conditions which would kill the unencysted or vegetative cells. Encystment is usually followed by two to several divisions of the nucleus, and when such multinucleate, mature cysts find themselves once more in favorable surroundings, multiple fission occurs and



several individuals emerge, each ready to engage in the activities of a feeding, growing form or **trophozoite**.

**AMOEBAE**

The common fresh-water amoeba, *Amoeba proteus*, is typical of the class Sarcodina. Lacking a limiting pellicle and specialized organelles, it appears under the microscope as a grayish to colorless irregular mass of granular, viscid material

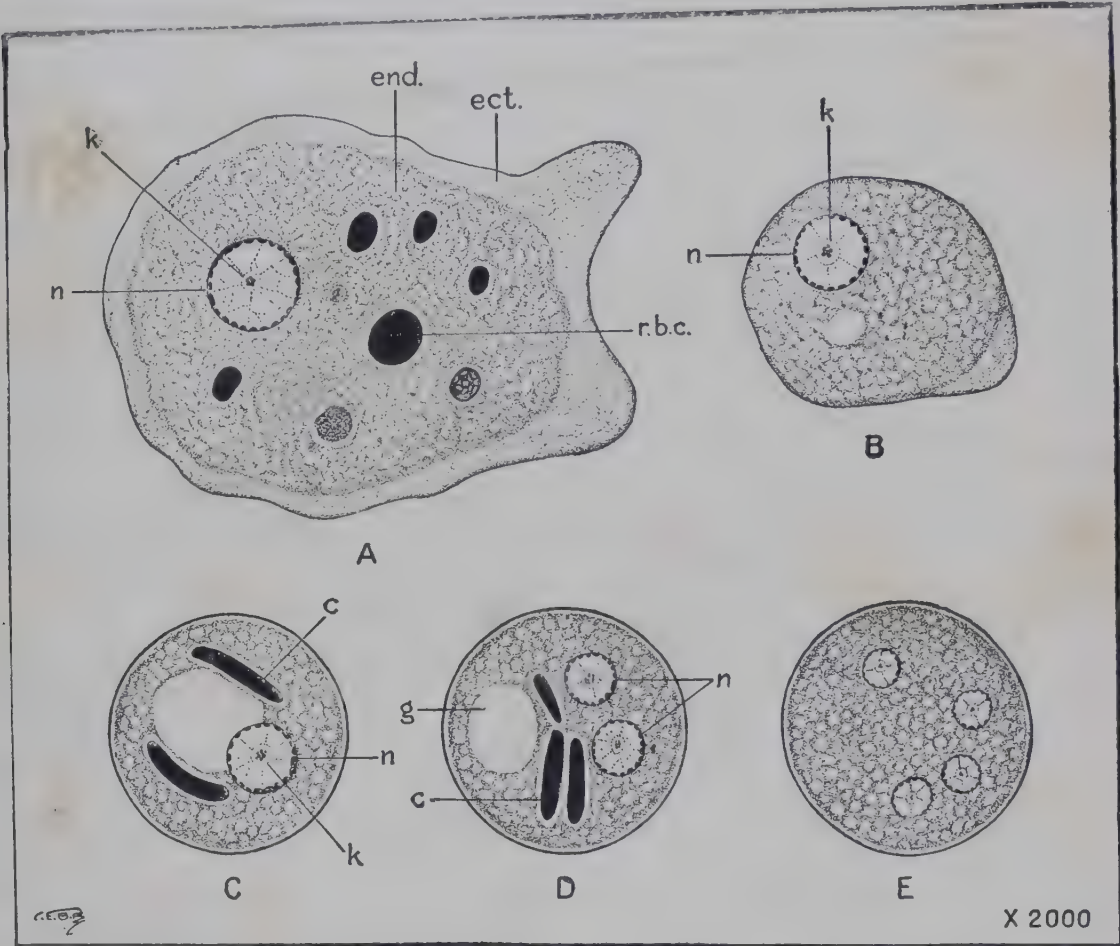


Fig. 12. *Endamoeba histolytica*. A, Trophozoite containing red blood cells which are being digested; B, precystic amoeba which has eliminated cytoplasmic inclusions; C, young uninucleate cyst; D, binucleate cyst; E, mature cyst with four nuclei.

c, Chromatoid body; ect., ectoplasm; end., endoplasm; g, glycogen vacuole; k, karyosome; n, nucleus; r.b.c., red blood cell.

(From Belding: *Textbook of Clinical Parasitology*, D. Appleton-Century Co., Inc.)

about 200 microns \* in diameter. Its shape changes from time to time as the protoplasm flows out to form the pseudopodia. This protoplasmic streaming or **amoeboid movement** is the means of locomotion and of capturing food. A pseudopodium may be formed anywhere on the surface of the cell by the pro-

\* A micron (often represented by the Greek letter *mu*,  $\mu$ ) equals 1/1000 of a millimeter or 1/25,000 of an inch.



trusion of the ectoplasm, followed by streaming of the granular endoplasm in the direction of the protrusion. The entire body may flow in one direction as it does in locomotion, or pseudopodia may be extended in more than one direction as in the capture of food. When the protoplasm contacts suitable food, a microscopic plant or animal, the pseudopodia flow around it to enclose it in a food vacuole. Thus food is ingested at any point on the cell's surface and several food vacuoles may be present. These together with the nucleus and a contractile vacuole change their position in the cell as they are carried along in the streaming cytoplasm. After the food has been digested and absorbed, any remaining indigestible material is expelled when the food vacuole reaches the surface of the cell. In the same way the contractile vacuole eliminates metabolic wastes and water from any point on the cell's surface. The amoeba reproduces by binary fission.

Other Sarcodina may be much smaller or larger than *Amoeba proteus*. Some free-living forms such as *Arcella* are covered by protective shells or tests. Certain parasitic amoebae live in the alimentary tract of man, the most common of these being *Endamoeba gingivalis* and *Endamoeba coli* which inhabit the mouth and large intestine, respectively, of many healthy persons. Three other harmless and fairly common intestinal amoebae are *Endolimax nana*, *Iodamoeba bütschlii*, and *Dientamoeba fragilis*. The only amoeba pathogenic for man is *Enda-*

*moeba histolytica* which causes amoebic dysentery. Most of these parasites form cysts, thick-walled, round to oval bodies smaller than the trophozoites of the same species. The nonpathogenic amoebae can be distinguished from each other and from *Endamoeba histolytica* by differences in the character of the nucleus, the size of the trophozoite and cyst, the number of nuclei in the mature cyst, the presence of elongated, deep-staining bodies, the chromatoid bodies, and other morphological features (Fig. 11). *Endamoeba gingivalis* and *Dientamoeba fragilis* do not form cysts.

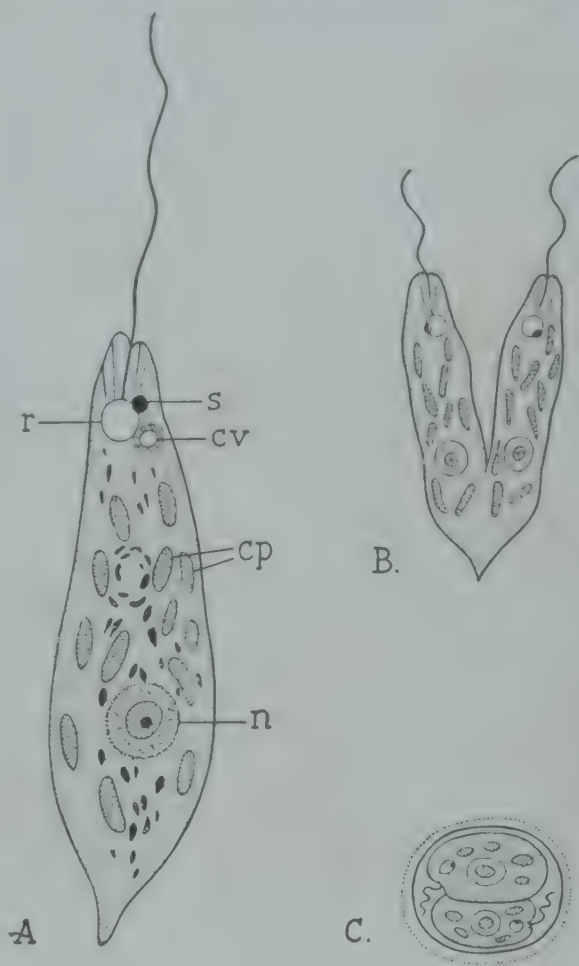


Fig. 13. *Euglena viridis*. A, Free-swimming individual showing structures: s, stigma; cv, contractile vacuole; r, reservoir; cp, chloroplast; n, nucleus. B, Reproduction by longitudinal binary fission. C, Binary fission within a cyst. (Redrawn from Mavor: *General Biology*, and Woodruff: *Foundations of Biology*, The Macmillan Co.)

## FLAGELLATES

A great number of highly diverse species compose the class Mastigophora, their common feature being the possession of one or more flagella. The typical flagellate is an oval, spindle or pear-shaped body whose form is maintained by a more or less tough flexible pellicle. The length of the cell may vary from 1 or 2  $\mu$  to about 200  $\mu$  in different species. Many of the free-living forms are

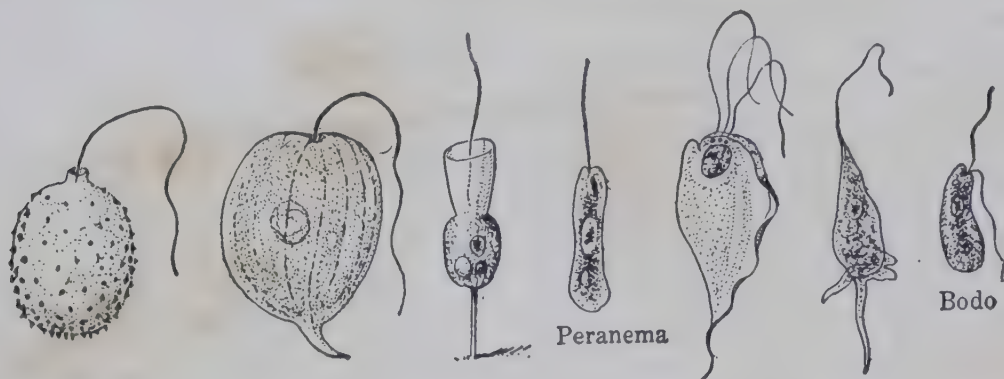


Fig. 14. Common flagellated Protozoa. From left to right: *Trachelomonas*; *Phacus*; *Monosiga*; *Peranema*; *Trichomonas*; *Cercomonas*; *Bodo*. (From Woodruff: *Foundations of Biology*, The Macmillan Co.)

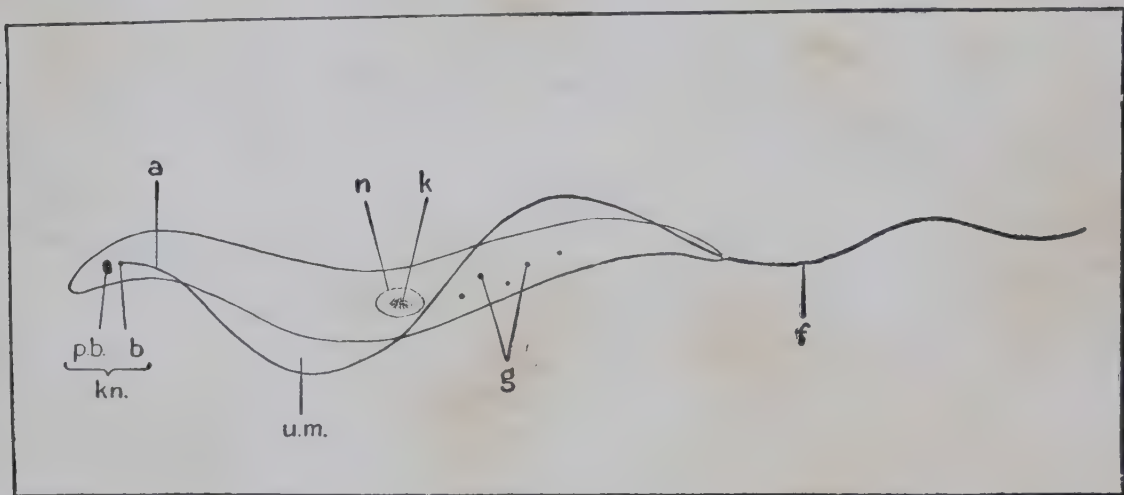


Fig. 15. Diagram of a typical trypanosome. a, Axoneme; b, blepharoplast; f, flagellum; g, chromatic granules; k, karyosome; kn, kinetoplast; n, nucleus; pb., parabasal body; u.m., undulating membrane. (From Belding: *Textbook of Clinical Parasitology*, D. Appleton-Century Co., Inc.)

colonial, encystment is common throughout the class and all reproduce by longitudinal binary fission.

Of the plant-like flagellates, *Euglena viridis* (Fig. 13) is a good example. This is a solitary organism equipped with a single flagellum which emerges at the anterior end of the spindle-shaped cell from a slight depression, the **cytostome** or cell mouth. The cytostome leads into a short **gullet**. Nearby a number



of small contractile vacuoles empty into a central vacuole which when full discharges its content through the gullet. A light-sensitive, red eye spot or stigma is also located in the anterior end of the cell. The nucleus is centrally placed and the cytoplasm contains a number of chlorophyll bodies, the chloroplasts. Nutrition is generally holophytic; the organism swims toward light in response to stimuli received by the stigma, and in the presence of light photosynthesis occurs. Dissolved foods are undoubtedly absorbed through the surface of the cell, and under

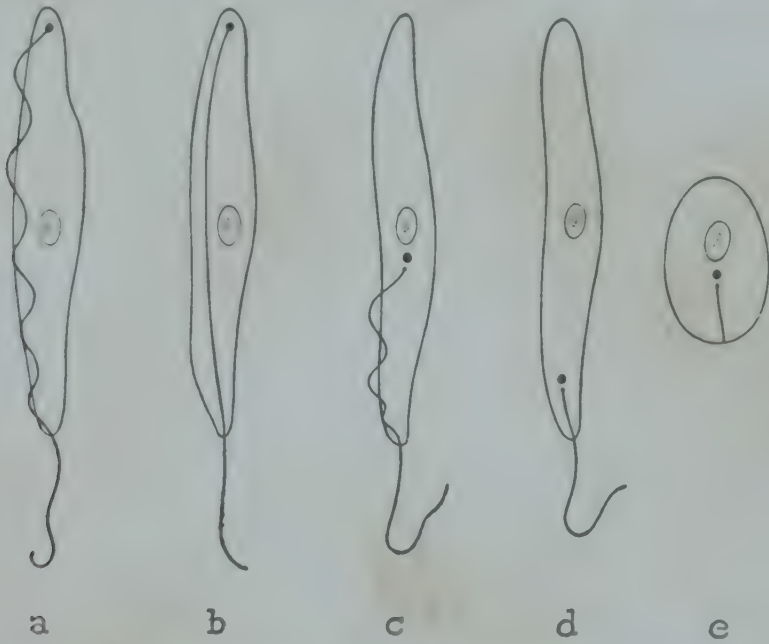


Fig. 16. Diagram of forms assumed by flagellates which parasitize blood and tissues (*Trypanosomidae*). a and b, Trypanosome type; c, crithidia type; d, leptomonas type; e, leishmania type.

*Trypanosoma* occurs as the trypanosome type (a) in vertebrate hosts and may assume any of the other forms (b, c, d, e) developing in invertebrate hosts and in cultures. *Leishmania* occurs as the nonflagellated type (e) in vertebrate hosts and develops into the leptomonas type (d) in invertebrates and in cultures. (Redrawn from Hegner, Root, Augustine and Huff: *Parasitology*, D. Appleton-Century Co., Inc.)

certain conditions particles of food, such as bacteria, may be ingested through the cytostome. Many green flagellates, such as *Volvox globator*, are colonial forms. Certain of these colonial green flagellates may render water unfit to drink by producing aromatic oils having a fishy odor and taste.

Flagellates without chlorophyll are characteristically animal-like in structure and physiology. Nutrition in the free-living forms is holozoic and saprophytic, i.e., by ingestion of organic food through a cytostome, and absorption of dissolved organic materials through the cell surface, respectively. Among the most complicated, as to cell structure and large numbers of flagella, are the wood-digesting flagellates which live symbiotically in the intestine of the termite. In *Bodo*, a typical fresh-water flagellate, two flagella emerge from the anterior end and one of these extends forward while the other bends backward, trailing behind the moving cell. In certain parasitic flagellates, such as the trypanosomes, a single flagellum

originates posteriorly but, instead of emerging from the cell in this region, it is directed anteriorly enveloped in a fold of the pellicle, the **undulating membrane** (Fig. 15). This membrane runs along one side of the elongated cell like a fish's fin and terminates at the anterior end where the flagellum emerges. The portion of the flagellum held within the cell is referred to as the **axoneme**. Inside the cell each flagellum originates from a deep-staining granule termed a **basal granule** or **blepharoplast**. One or more basal granules may lie close to a larger **parabasal body** and these structures are associated with the control of flagellar contractions and coordination of movement. The position of the basal granule is a distinguish-

	CHILOMASTIX MESNILI	TRICHOMONAS HOMINIS	EMBADOMONAS INTESTINALIS	ENTEROMONAS HOMINIS	GIARDIA LAMBLIA
TROPHOZOITE					
CYST		UNKNOWN			

Fig. 17. Intestinal flagellates of man. a, Axostyle; b, blepharoplasts; c, cytotome; f, flagella; n, nucleus; s, shields; s.g., spiral groove; u.m., undulating membrane. (From Belding: *Textbook of Clinical Parasitology*, D. Appleton-Century Co., Inc.)

ing characteristic between types of flagellates that parasitize the blood and other tissues of man and animals. In the life cycle of each of these parasites two or more types occur. For instance, it will be noted (Fig. 16) that a flagellum is lacking in the so-called leishmania type, but this same organism growing under different conditions develops a flagellum. *Trypanosoma* and *Leishmania*, the two important pathogenic genera which parasitize the blood and tissues of man and animals, will be discussed in connection with the diseases caused by them (Chapter 42).

Certain Mastigophora commonly parasitize the alimentary tract of men and



animals, but most of these are harmless or of doubtful pathogenicity. For example, *Trichomonas buccalis* is frequently found in the mouth while *Trichomonas hominis* and *Chilomastix mesnili* inhabit the large intestine with no clinical symptoms. However, *Trichomonas vaginalis* is often associated with a vaginitis, and there is fairly good evidence that one intestinal flagellate, *Giardia lamblia*, may cause an enteritis accompanied by a persistent diarrhea. Compared to that of other flagellates the structure of *Giardia* and the trichomonads is quite complicated (Fig. 17). In addition to structures already described, these organisms contain stiff supporting rods or **axostyles** which may extend posteriorly beyond the cell body.

## CILIATES

The common free-living ciliate *Paramecium caudatum* is typical of the class Infusoria. The slipper-shaped cell is about  $200\ \mu$  long and is entirely covered with rows of cilia. Embedded in the ectoplasm just below the cilia are spindle-

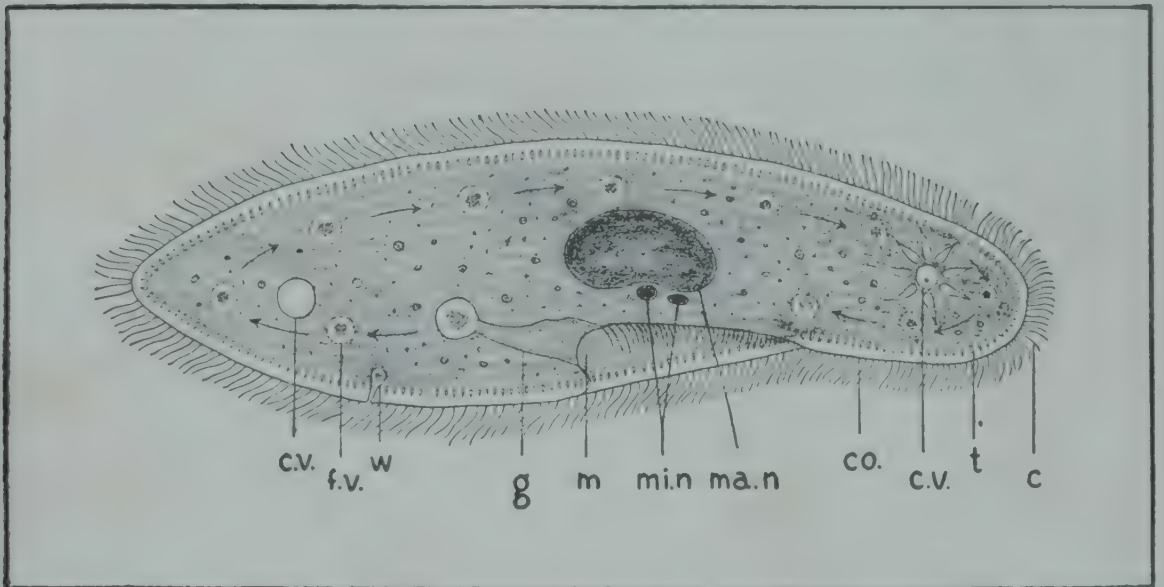


Fig. 18. Diagram of *Paramecium*. c, Cilia; co., cortex; c.v., contractile vacuole; f.v., food vacuole; g, gullet; m, mouth; ma.n., macronucleus; mi.n., micronucleus; t, trichocyst; w, waste being egested through anal spot. (From Belding: *Textbook of Clinical Parasitology*, D. Appleton-Century Co., Inc.)

shaped sacs, the **trichocysts**, which on stimulation discharge entangling threads that serve as protective weapons. An oral groove or **peristome** follows an oblique course half the length of the cell from the blunt anterior end to a cytostome which opens into a funnel-shaped gullet. A row of heavy cilia along the peristome and an undulating membrane in the mouth region sweep whatever food lies in the animal's path through the mouth and into the gullet. Food vacuoles form at the end of the gullet and are moved around the cell by **cyclosis**, the circulation of the cytoplasm. This circular course finally carries each food vacuole to the anal

spot or **cytopyge** where indigestible material is egested. Two contractile vacuoles, one at each end of the cell, alternately contract and expand as **radiating canals** fill first one and then the other with metabolic wastes. The paramecium turns on its long axis as it is moved forward through the water by the backward stroke of its cilia; reversal of the stroke drives the organisms in the opposite direction. A **neuromotor apparatus** consisting of a system of fibers connects the basal granules of the cilia and coordinates their motion. A large **macronucleus** which controls metabolic activities and a small **micronucleus** governing reproductive

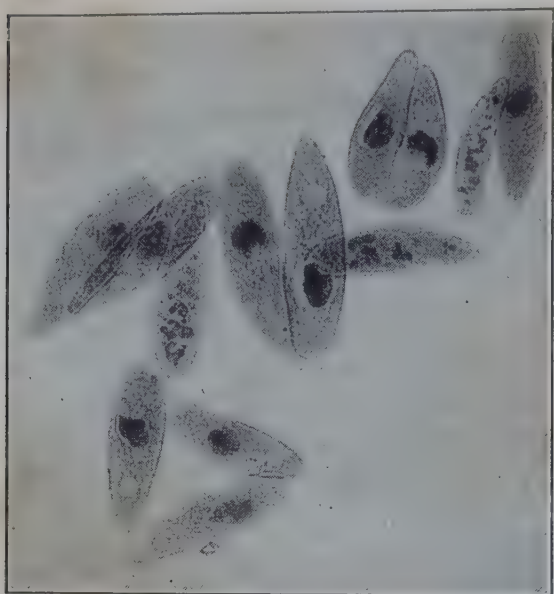


Fig. 19. Conjugating paramecia. (Courtesy of General Biological Supply House, Inc., Chicago.)

processes are located at the center of the cell. Reproduction is usually accomplished by transverse binary fission. After a number of asexual generations nuclear reorganization is accomplished sexually by conjugation, two individuals uniting temporarily to exchange nuclear material, or by a process known as **endomixis** in which the changes in the individual are similar to those of conjugation except that no foreign nuclear substance is introduced.

Other free-living ciliates may vary from 10  $\mu$  to 2 mm. in length, may possess more than two nuclei, and instead of swimming free they may creep on leg-like **cirri** (sing., **cirrus**) formed by fusion of groups of cilia. Stalked individuals such as *Vorticella* are sessile and

some close relatives live in colonies. Certain species are parasites of vertebrate and invertebrate animals, but only one ciliate is encountered in man. This is *Balantidium coli*, a large cyst-forming intestinal parasite which occurs commonly in hogs and infects man occasionally to cause ciliary dysentery or **balantidiasis** (Fig. 21).

## SPOROZOA

Protozoa included in the class Sporozoa are adapted to a strictly parasitic existence. They are degenerate in that they have no locomotor organelles, mouth, anal opening or other structures essential to the free-living forms. They are nourished by substances absorbed from the body fluids or cells of a host organism. On the other hand, their reproductive capacity is greater than that of most other protozoa and their life cycle is more complicated. After several generations of multiple fission (schizogony) gametes are produced. By union of a mature male and female gamete a zygote is formed which may develop into a resistant walled spore subsequently containing one or more elongated bodies,



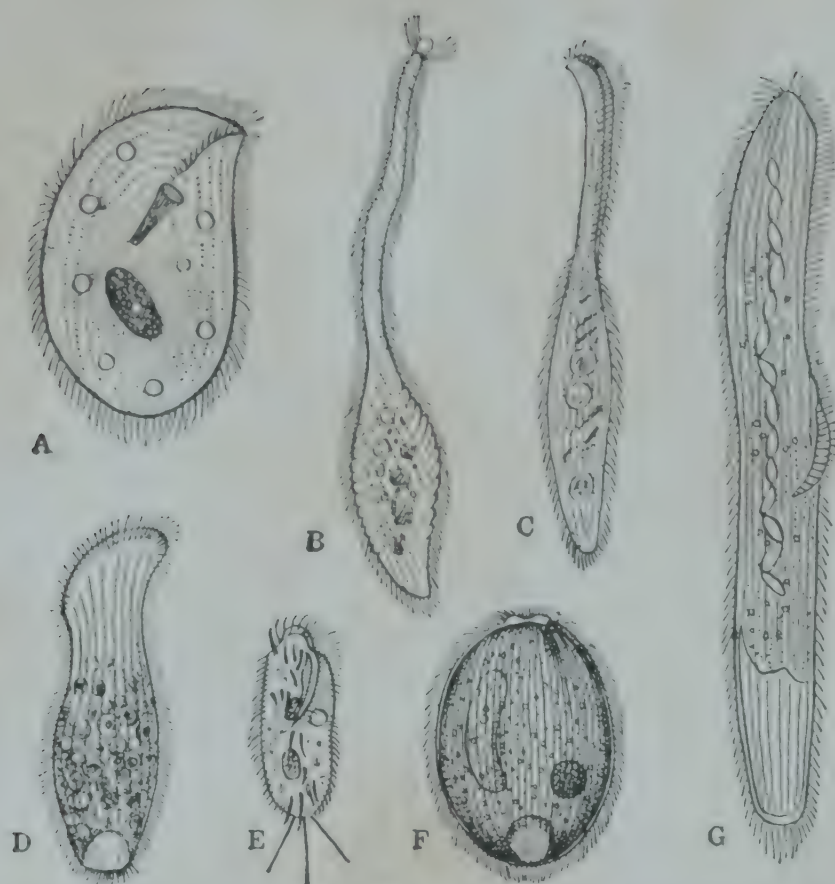


Fig. 20. Some common ciliates from fresh water. A. *Chilodonella*; B. *Lacrymaria*; C. *Lionotus*; D. *Spathidium*; E. *Stylonychia*; F. *Prorodon*; G. *Spirostomum*. (From Woodruff: *Foundations of Biology*, The Macmillan Co.)

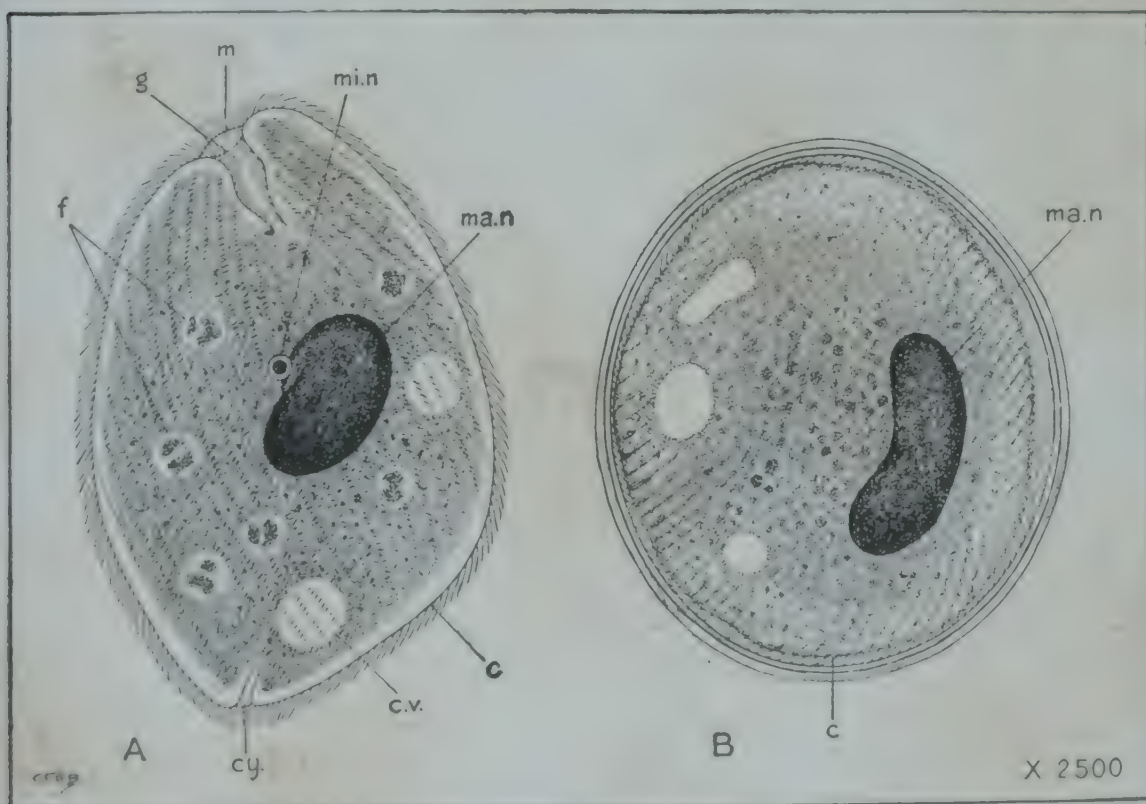


Fig. 21. *Balantidium coli*. A. Trophozoite; B. cyst; c. cilia; cy., cytophyge; c.v., contractile vacuole; f, food vacuole; g, gullet; m, mouth; ma.n., macronucleus; mi.n., micronucleus. (Modified from Dobell and O'Connor, in Belding: *Textbook of Clinical Parasitology*, D. Appleton-Century Co., Inc.)

the **sporozoites**, or into a number of naked sporozoites. The zygote thus initiates multiplication by spore formation or sporogony. In the typical life cycle sexual and asexual reproduction occur alternately in the same or in two different hosts. In the case of the malarial parasite the generation produced by sexual reproduc-

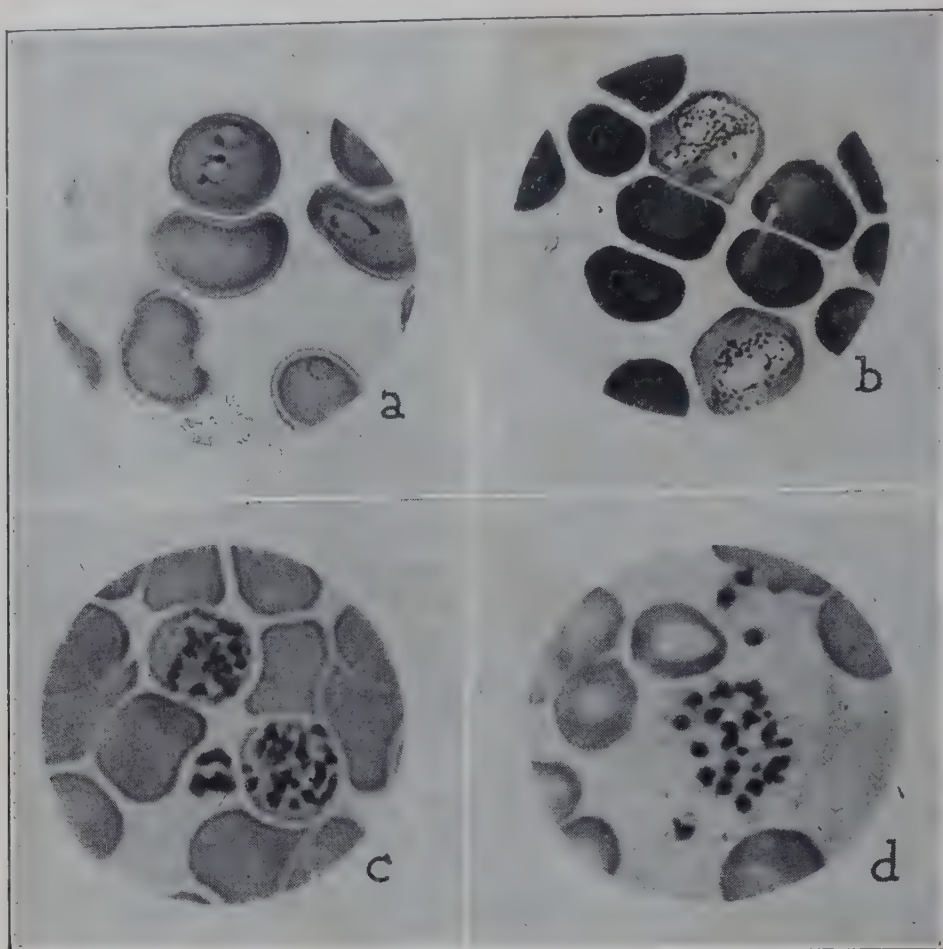


Fig. 22. Asexual reproduction of malarial parasites in red blood cells. a, Young, ring forms of *Plasmodium falciparum* trophozoites; b, growing forms or schizonts of *Plasmodium vivax*; c, segmenting schizont of *Plasmodium malariae*; d, release of segments or merozoites of *Plasmodium vivax* from a red blood cell. Each merozoite can infect a red blood cell and repeat the asexual cycle. (Photomicrographs from the Army Institute of Pathology, Neg. No. 30843.)

tion develops in an invertebrate host, a mosquito, and is followed by generations produced asexually in a vertebrate host such as bird or man.

Practically all kinds of animals may be infected by one or more species of Sporozoa. Many of these are harmless parasites but some may cause severe disease. The most important for man are the malarial parasites *Plasmodium malariae*, *P. vivax*, *P. falciparum* and *P. ovale* which multiply inside the red blood cells and are therefore included in a subgroup named *Haemosporidia*. These organisms will be described in the discussion of malaria (Chapter 42). One species of Sporozoa is known to parasitize the epithelial cells lining the human intestine; this is *Isospora hominis*, a member of the Order *Coccidia*. **Coccidiosis**,





Fig. 23. Sporozoites of *Plasmodium* in salivary gland of mosquito and in surrounding fluid. (From Mackie, Hunter, and Worth: *A Manual of Tropical Medicine*, W. B. Saunders Co.)

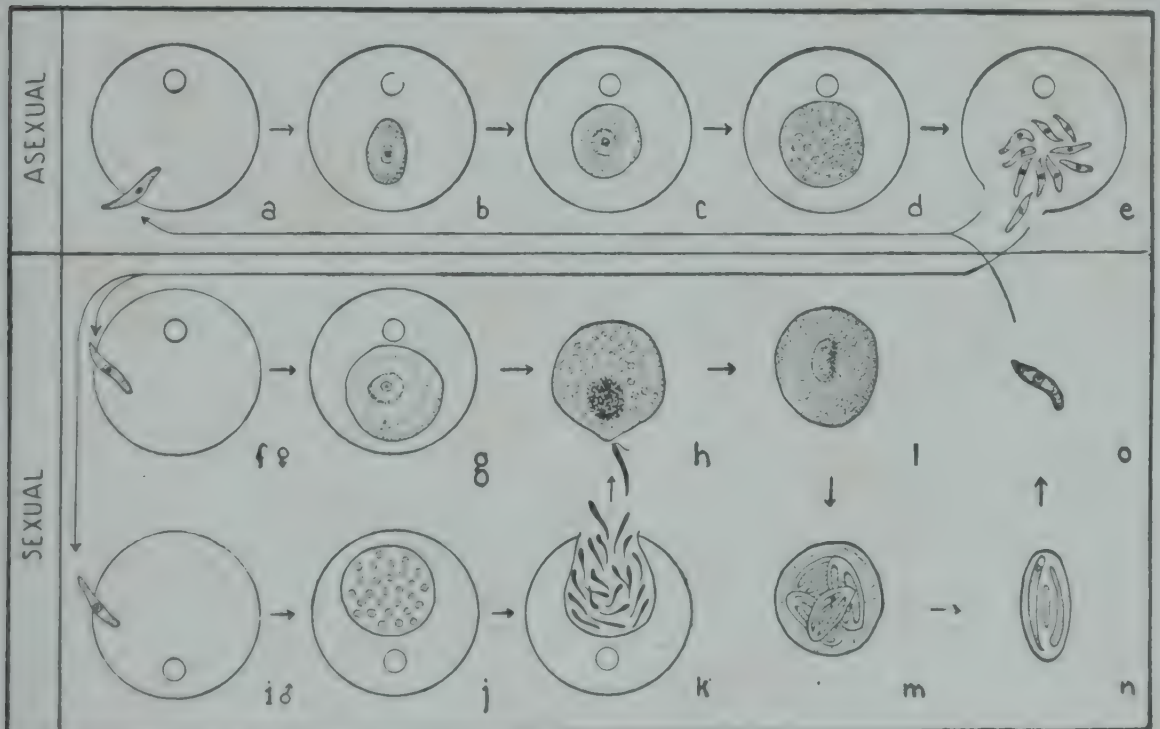


Fig. 24. Life cycle of a typical coccidium. a. Sporozoite invading cell; b, trophozoite developing in cell; c, schizont; d, mature schizont; e, merozoites erupting from cell; f to h, development of macrogamete (female sex cell); i to k, development of microgametes (male cells); l, union of macro- and microgametes; m, resulting oocyst with spores; n, spore with sporozoites; o, sporozoite liberated from spore. (From Belding: *Textbook of Clinical Parasitology*, D. Appleton-Century Co., Inc.)

a disease characterized by diarrhea, is rare in man but a serious and prevalent infection in domestic and wild animals. *Coccidia* multiply in the intestine and produce cysts which are carried outside the body in the feces. Spores develop inside each cyst and they in turn produce sporozoites. The disease is transmitted through food or water contaminated with feces containing the infective cysts. Thus the life cycle of *Coccidia* is passed in a single host and in the environment (Fig. 24).

Intracellular parasites named *Toxoplasma* are generally considered with the Sporozoa although it is not even certain that they are protozoa. First recognized as a disease entity in 1939, **toxoplasmosis** has been observed as an acute encephalitis in children and a rare febrile exanthematic disease resembling typhus and spotted fever in adults. By 1948 twenty-seven cases of toxoplasmosis had been confirmed by autopsy.



# 4

## THE ALGAE

The simplest plants belong to the *Thallophyta*, a great group possessing no true roots, leaves or stems. Their plant body or **thallus** lacks the structural differentiation of the higher forms, the mosses, ferns and seed-bearing plants. Members of the *Thallophyta* fall into two subgroups, the *Algae* and the *Fungi*, depending on whether or not they contain the green pigment chlorophyll. The algae are chlorophyll-bearing, whereas the fungi are devoid of this material and are generally referred to as the colorless plants.

The algae are chiefly aquatic and are widely distributed in both fresh and sea waters. Those that live on land grow only where there is abundant moisture. In size and structure they range from single-celled organisms through a variety of microscopic colonial forms to large multicellular plants such as the giant seaweeds. *Protococcus*, which grows on the north side of tree trunks and covers moist shaded soil, stones and flower pots with a green "mossy" coat, the green "pond scum" of stagnant waters and the seaweeds are familiar algae. The aquatic algae may be inactive floating forms, actively motile microscopic organisms, or fixed plants attached to rocks by root-like "hold fasts."

Algae sometimes live symbiotically with other plants; for example, lichens are an association of algae and fungi, or with certain animals, as in the case of the alga *Chlorella* living in *Hydra viridis*. The great majority are independent free-living organisms. They contribute indirectly to man's welfare by serving as food for many aquatic animals and as important sources of such materials as gelatin, agar-agar and diatomaceous earth, a substance used in commercial polishing powders and fine-pored filters. At certain times of the year prodigious numbers

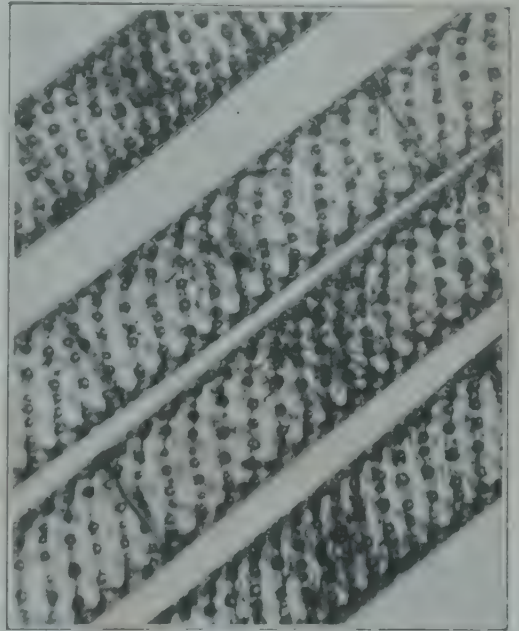


Fig. 25. Highly magnified cells of the filamentous green alga *Spirogyra*. ( $\times 100$ .) Masses of this and other filamentous algae form the "pond scum" of stagnant waters. (Photographed by W. H. Simmons.)

of certain blue-green algae may appear in water, imparting to it an obnoxious taste and odor. The blue-green algae are of special interest to the bacteriologist because they seem to bear a close relationship to bacteria.

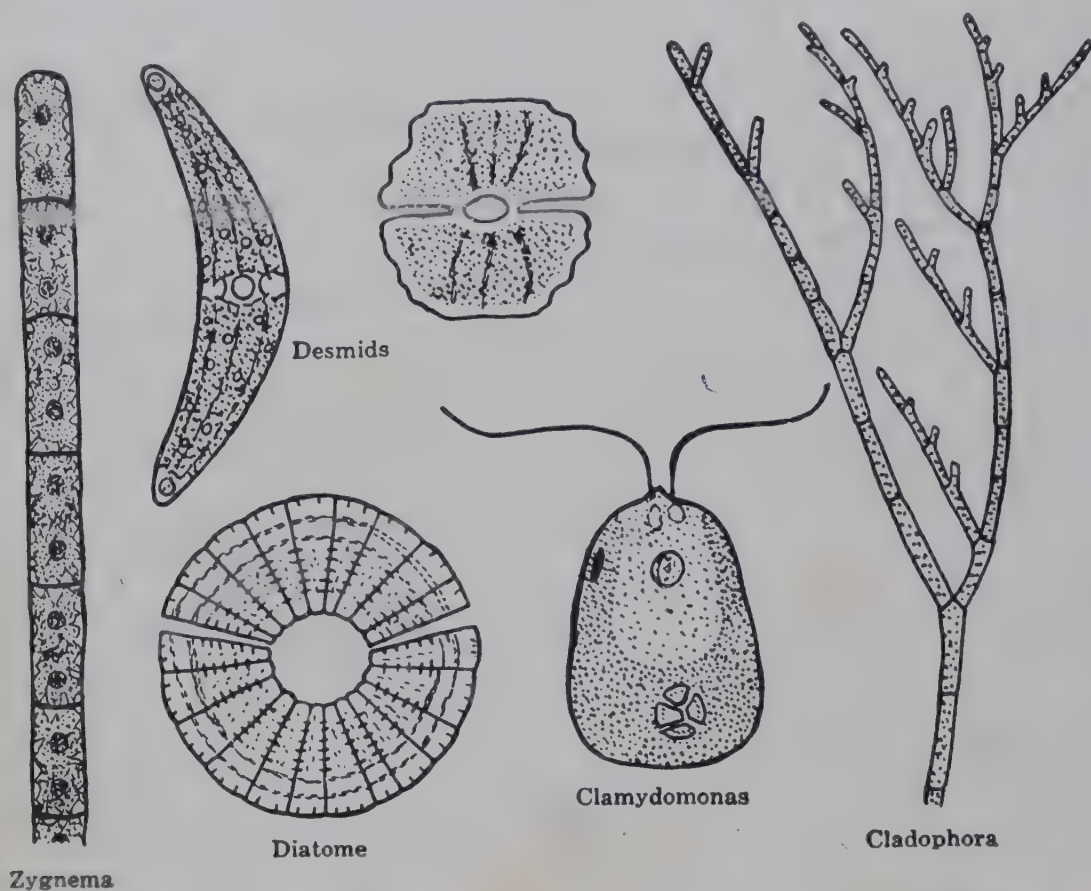


Fig. 26. Various green algae and a diatom illustrating unicellular, flagellated, and multicellular, filamentous forms. (From Mavor: *General Biology*, The Macmillan Co.)

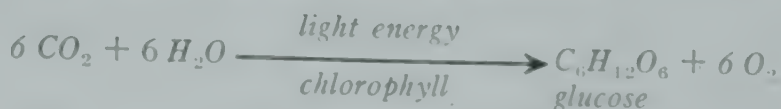
**Classes of Algae.** As previously stated, all algae contain chlorophyll. In addition many possess other pigments which mask or modify the green color. According to their pigmentation the algae are subdivided into groups or classes as follows:

- Myxophyceae* or blue-green algae
- Chlorophyceae* or green algae
- Phaeophyceae* or brown algae
- Rhodophyceae* or red algae
- Chrysophyceae* or yellow algae

**Green Plant Nutrition.** The algae, like all green plants, live by **holophytic** nutrition, *i.e.*, they take in inorganic substances, mineral salts, water and carbon dioxide, and from these simple materials synthesize their foods. The basic step in this conversion of inorganic compounds into the complex carbohydrates, fats and proteins is **photosynthesis**, a process by which carbon dioxide and water, in the presence of light and chlorophyll, are transformed into sugar and oxygen.



Although photosynthesis involves more than one chemical reaction the summary of events may be represented by the following equation:



Generally the sugar thus formed is rapidly changed to starch, a more complicated and insoluble carbohydrate suitable for storage in plant cells. Before utilization the starch is changed back into the original soluble sugar. Sugar may be used

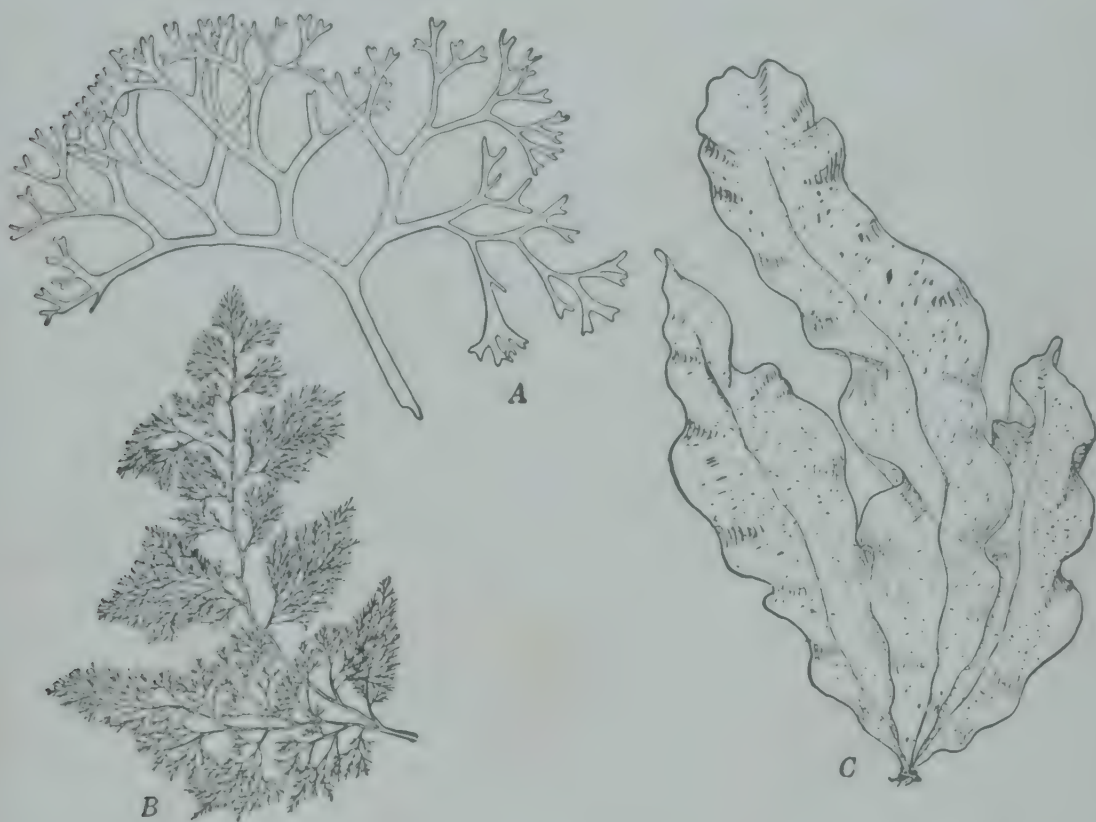


Fig. 27. Types of red algae, seaweeds which inhabit the oceans below tide level. (From Smith, et al.: *A Textbook of General Botany*, The Macmillan Co.)

in two chief ways: it may be oxidized to supply energy, or it may be converted into protoplasm. The first process, by which energy is released, is known as **respiration**. In the second, sugar may be built into more complex substances such as the cellulose of the cell wall or the carbohydrate, lipid and protein constituents of the protoplasm. The exact mechanisms by which the simple sugar is converted into fats and proteins are not known. Carbohydrate and fat molecules contain the same elements, carbon, hydrogen and oxygen, in differing proportions and chemical arrangements, and the plant has some efficient mechanism for performing the conversion. For protein synthesis the green plants require a variety of mineral salts, including nitrates, sulfates and phosphates, which are combined with the carbohydrate products of photosynthesis. The nitrate ( $-\text{NO}_3$ ) radical

of common nitrate salts, such as sodium nitrate ( $\text{NaNO}_3$ ), potassium nitrate ( $\text{KNO}_3$ ) and calcium nitrate [ $\text{Ca}(\text{NO}_3)_2$ ], is changed to an amine group ( $-\text{NH}_2$ ) which is an essential part of every amino acid. Many different amino acids are thus made available and are united to construct the variety of protein molecules needed by the cells.

**Green Plant Respiration.** Respiration in the algae and all green plants is fundamentally the same as in other living organisms. The food within the cells is broken down by a series of oxidative reactions leading to a release of energy and

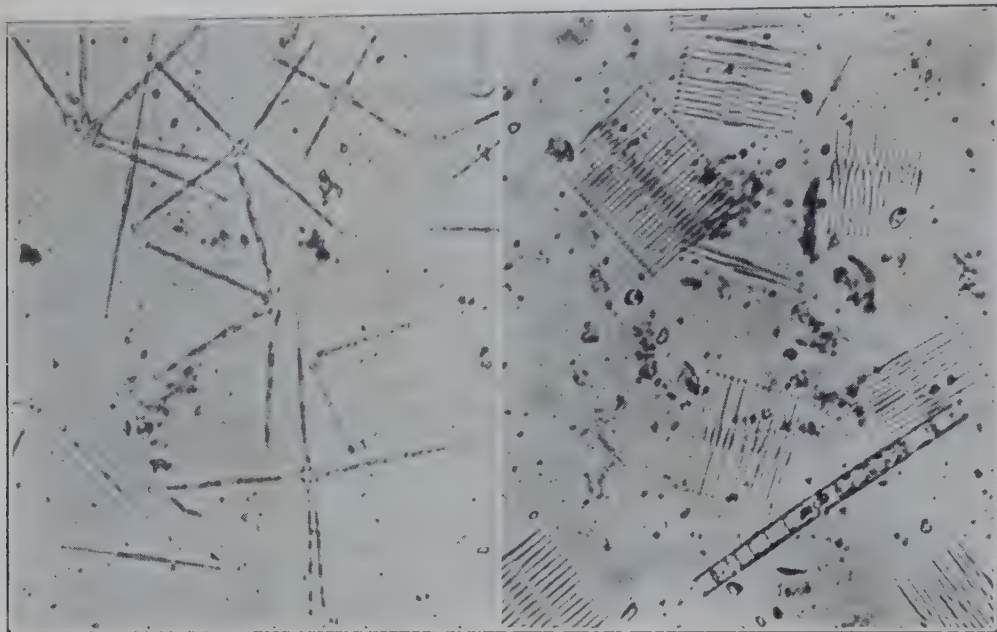
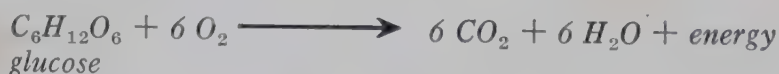


Fig. 28. Diatoms, common unicellular algae of fresh-water and marine plankton. Left, *Asterionella*,  $\times 100$ . Right, *Synedra pulchella*,  $\times 165$ . (Photographs by W. H. Simmons. Courtesy of Phelps Dodge Refining Corp.)

waste products. The complete oxidation of sugar, which is the most easily available source of energy, requires gaseous oxygen and results in the formation of carbon dioxide and water. The following equation summarizes the complete oxidation of glucose.



In the cells of green plants there is a continuous exchange of the gases oxygen and carbon dioxide associated with the energy-releasing process. The green plants cannot manage long without free oxygen, for they, like the great majority of animals, are aerobic organisms.

**Practical Contributions of Algae to Microbiology.** Agar-agar, a polysaccharide which is used to prepare liquefiable solid culture media for growing the microscopic fungi, is derived from certain seaweeds belonging to the red algae. Yellow algae known as diatoms have also contributed to microbiological methods. These common unicellular algae (Fig. 28) secrete a wall of hard silicious material,



an exoskeleton. Through the ages great deposits of this material in the form of fossils have accumulated on the bottom of oceans and fresh water bodies, and may be found in some regions of the continents which were once submerged. The



Fig. 29. Blue-green algae. Left, *Anabaena*, *Aphanizomenon*, *Coclosphaerium* and *Clathrocystis*.  $\times 200$ . Right, *Oscillatoria*.  $\times 100$ . (Photographs by W. H. Simmons. Courtesy of Phelps Dodge Refining Corp.)

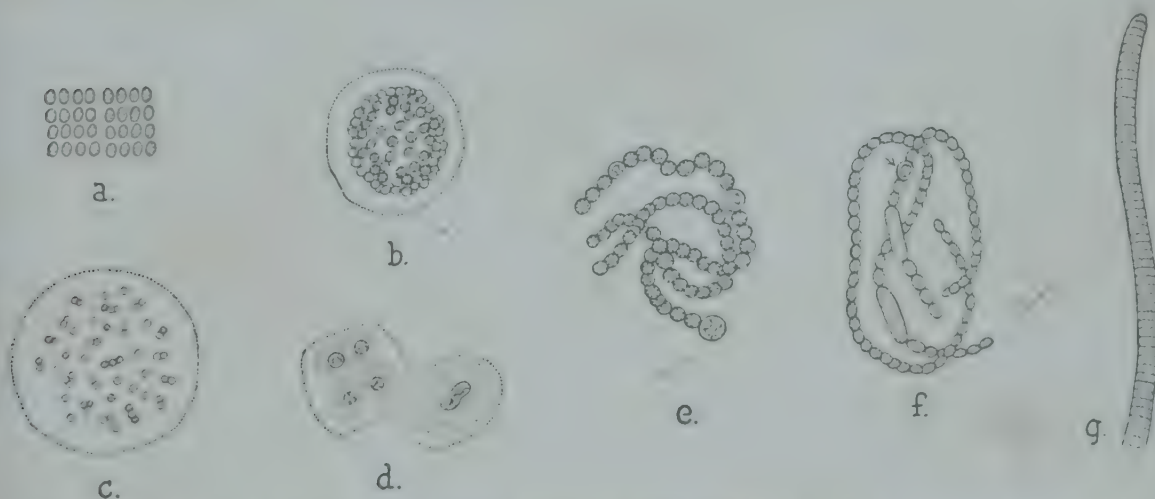


Fig. 30. Various blue-green algae showing single-celled forms adhering together in colonies often surrounded by a gelatinous mass and filamentous forms. a. *Merismopedia*; b. *Coclosphaerium*; c. *Aphanocapsa*; d. *Gloeocapsa*; e. *Nostoc*; f. *Anabaena*; g. *Oscillatoria*. (Redrawn chiefly from Smith, Gilbert, et al.: *A Textbook of General Botany*, The Macmillan Co.)

white, silicious powder found in these regions is known as diatomaceous earth or by the German name *Kieselguhr*. It has a number of commercial uses including the manufacture of fine-pored filters employed by microbiologists in the study of the metabolic products of microorganisms and of the filtrable viruses. The

value of these filters will become clear in further discussions, particularly of the viruses and bacterial exotoxins.

**Similarities of the Blue-Green Algae and Bacteria.** The blue-green algae are the most primitive of green plants. They are unicellular round to oval forms occurring singly or, more commonly, in chains, filaments, or colonies surrounded by gelatinous capsules or sheaths (Fig. 30). In addition to chlorophyll they contain other pigments, including **phycocyanin** which gives most of them a bluish color. The organization of the protoplasm in each cell points to their primitive nature. There is no well defined nucleus. Simple binary fission is the common method of reproduction. Instead of being enclosed in specialized bodies, the chloroplasts, as it is in the cells of other green plants, the chlorophyll is distributed throughout the protoplasm. Carbohydrates are stored in the form of glycogen, or a glycogen-like compound, as they are in animals and in the fungi. The lack of a distinct nucleus, the absence of chloroplasts, the simple method of reproduction and general morphology suggests a close relationship to the bacteria. However, the larger size of the blue-green algae and their possession of chlorophyll sharply distinguish them from bacteria.



# 5

## THE FUNGI

Fungi are chlorophyll-free plants which have no true roots, leaves or stems. The so-called true fungus or **eumycete** characteristically possesses a body composed of a web of thread-like elements known as a **mycelium** and reproduces by spores. The eumycetes include large fleshy fungi such as mushrooms, toadstools, puffballs and the wood-loving bracket fungi, as well as the microscopic rusts, smuts, mildews, molds and yeasts. Of these only the yeasts are unicellular. While bacteria are colorless plants they generally do not develop a mycelium and do not produce reproductive spores. For these and other reasons they, along with the

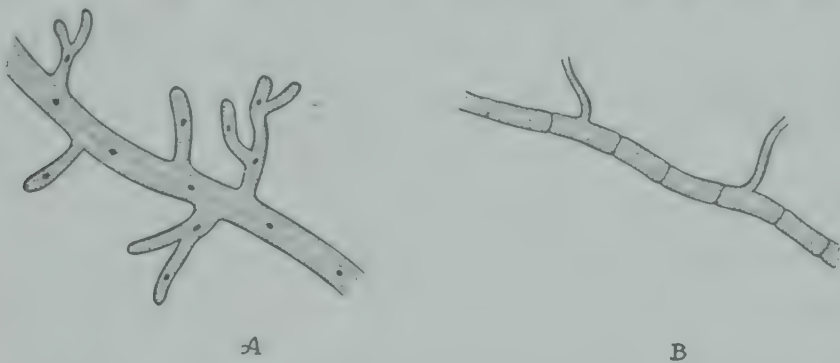


Fig. 31. A. Non-septate or coenocytic mycelium. B. Septate mycelium.

slime molds (Myxomycetes), which have certain features in common with both animals and fungi, are considered to be false fungi (pseudomycetes). In medical microbiology the term *fungus* is reserved for the yeasts and molds, and whereas mycology is the study of any or all fungi, medical mycology is restricted to the study of parasitic yeasts and molds.

Since the fungi have no chlorophyll, they are unable to utilize sunlight in photosynthesis as are the green plants and thus, with rare exceptions, they are obliged to adopt a **saprophytic** or **parasitic** mode of life. A saprophyte, or generally a "rot plant," decomposes dead organic matter outside its body and subsequently absorbs and utilizes the digested products for food. The majority of fungi are saprophytes since they live by decomposing the wastes and dead bodies of plants and animals in soil and water. Foods intended for human consumption are often spoiled, or digested and used, by yeasts and molds before man has a chance to avail himself of them. So adaptable are these colorless plants that

some kinds can grow on almost any organic matter, even on fabrics, leather and paper, so long as sufficient moisture is present. It is not difficult to imagine how certain varieties can establish themselves on the nonliving outer surfaces of plants and animals, on the surfaces of leaves and stems of plants and on the superficial layers of skin, the nails and hair of animals. Considering the huge numbers and variety of fungi in the world, relatively few kinds are able to exist within the bodies of living plants and animals. Some of these parasitic fungi produce disease, as will be discussed in a later section. In addition, the fungi are of great importance in the decomposition of organic matter, thus making available substances essential to the nutrition of green plants and, indirectly, to that of animals. In some instances they are direct sources of food for man and animals.

**The Structure of Fungi.** The typical fungus has a **vegetative mycelium** growing down into the substratum from which it secures its food, and an aerial, **reproductive mycelium** specializing in the production of spores. Each thread of the mycelium or **hypha** is composed of many cylindrical-shaped cells arranged end to end. In the aerial portion of some fungi, such as mushrooms, these hyphae are bundled together into a compact mass, but beneath the surface of the soil there is an extensive filamentous mycelium. In the most alga-like fungi, the *Phycomycetes*, the cells forming the hyphae are not separated by cross walls or **septa**, but each hypha is a continuous tubular filament of protoplasm containing many nuclei (Fig. 31). Such a mycelium is said to be **nonseptate** or **coenocytic**. The other multicellular fungi have a **septate** mycelium in which the individual cells of the hypha are separated by cross walls. The cell walls of the true fungi, including the yeasts and molds, are relatively thick and rigid. Their exact chemical composition has not been determined, but they are not made of cellulose as are the cell walls of the typical green plants. Special staining techniques reveal one or more small nuclei in each cell. The unstained cells of a typical fungus appear granular and contain several kinds of vacuoles which by appropriate stains may be shown to be composed of food materials, fat globules, glycogen granules and a deep-staining or **metachromatic**, nitrogenous substance known as **volutin**.

**Classification of the Fungi.** The fungi are classified principally according to the type of reproductive spores they produce and whether they possess a septate or a coenocytic mycelium. The six classes of fungi and their distinguishing characteristics are summarized below.

*Eumycetes* (true fungi)

*Phycomycetes* —molds having coenocytic mycelium; the familiar *Phycomycetes* reproduce asexually by **sporangiospores** and sexually by **zygospore** formation.

*Ascomycetes* —yeasts, molds and some fleshy fungi reproducing sexually by **ascospores**; septate mycelium; asexual reproduction by free spores or **conidia**.

*Basidiomycetes* —fungi producing **basidiospores**; most are large fleshy fungi such as mushrooms, puffballs and bracket fungi; rusts and smuts which parasitize plants, and a few yeasts belong here; none are pathogenic for man and animals.



*Hyphomycetes* —a “waste basket” group of yeasts and septate molds in which (Fungi no sexual spores have been found; most fungi pathogenic Imperfecti) for man are in this class.

*Pseudomycetes* (false fungi)

*Myxomycetes* —“slime molds”; free-living organisms existing as motile amoeboid masses in one stage and as sessile, spore-bearing, fungus-like forms in another stage of their life cycle. The spores develop into the amoeboid form. Zoologists name these **Mycetozoa** or fungus animals.

*Schizomycetes* —the fission fungi; the bacteria belong in this class since they are unicellular, chlorophyll-free and reproduce by binary fission.

It will be noted that four of the six classes are eumycetes. Of these only the microscopic organisms, the yeasts and molds, will be considered in this chapter. Some of the important parasitic fungi will be more fully discussed later in the text (Chapter 41).

## THE MOLDS

The filamentous fungi known as molds are widely distributed throughout the world in soil and water and as parasites of plants and animals. They need no introduction, for practically everyone has encountered their cottony or velvety growths at one time or another. However, neither the hyphae that burrow into the nutrient substrate and spread the patch of mold nor the huge numbers of spores on the aerial hyphae are ordinarily visible without microscopic examination. Close inspection shows that the mature, spore-bearing heads of the common molds are generally colored, whereas the unripe spores and often the mycelium are white or colorless. The ripe buoyant spores are blown by the hundreds from the tops of the mold plants and are spread far from the parent plants by air currents. Indeed the spores of the fungus may be carried thousands of miles in this way. Gradually they settle out of the air into the soil, water, dust and on all surfaces. If the new habitat is suitable the spore will germinate and reproduce a mold plant similar to the parent. Molds are famous for their ability to adapt themselves to a wide variety of conditions. In an unfavorable environment the spores survive for considerable time but do not germinate.

**Phycomycetes.** The most primitive of the eumycetes are molds belonging to the class Phycomycetes or the alga-like fungi. Members of this group are wide-spread in nature, growing in water, soil and on all sorts of decaying organic matter. Some are important parasites of land and water plants as well as of aquatic animals. The water mold *Saprolegnia* not uncommonly infects goldfish and minnows kept in small vessels of water. The Phycomycetes encountered, often as airborne contaminants, in the bacteriology laboratory generally belong to the genus *Rhizopus* or *Mucor*.

**Rhizopus.** The molds of this group frequently appear as a white or gray fuzzy growth on bread or other starchy foods. The vegetative portion of the co-

enocytic mycelium develops root-like structures, the **rhizoids**, and **stolons** or runners that push out from the plant parallel to the surface of the subsoil and germinate new plants at their tips in much the same way as strawberry plants are spread by runners. In a laboratory plate culture *Rhizopus* quickly covers the surface of the medium, climbs the sides of the dish and may even grow on the lid of the dish clinging to the glass by its rhizoids (Fig. 34). A good way to study this mold is to remove the lid from such a culture and examine it under the low-power objective of the microscope. The aerial mycelium is composed of hyphae

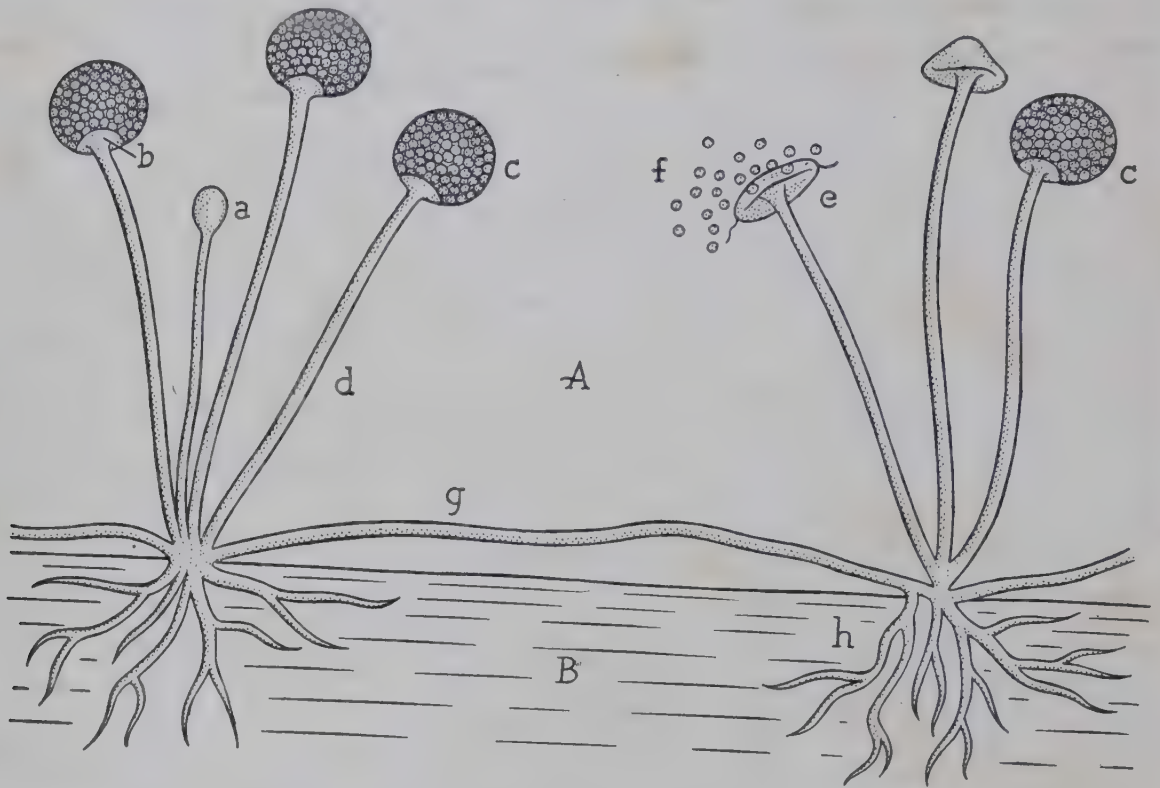


Fig. 32. *Rhizopus nigricans*. A, Reproductive mycelium: a, immature sporangium; b, columella; c, mature sporangium filled with sporangiospores; d, hypha bearing sporangium or sporangiophore; e, broken sporangium leaving collapsed columella; f, sporangiospore. B, Vegetative mycelium: g, stolon; h, rhizoids embedded in nutrient substrate.

which develop asexual spores named **sporangiospores** because they are formed inside a spore case or **sporangium** (Fig. 32). The erect hypha bearing the sporangiospores is known as a **sporangiophore**. Clusters of sporangiophores and rhizoids form at the same point on the stolon. The immature sporangium first appears as a colorless terminal swelling of the aerial hypha. The protoplasm in this enlarging tip increases and finally divides into numerous individual cells, the spores, which when ripe have a spore wall and brown or black pigmentation. Protruding up into the sporangium is the **columella**, a hemispherical cell which separates the spore-bearing head from the rest of the hypha and by the pressure it exerts guarantees the rupture of the mature spore case. The ripe spores thus liberated are widely disseminated by air currents. Under proper conditions each



spore germinates into the mycelium of a new mold plant. This asexual method is the common way of reproduction, but under certain conditions, rarely simulated in laboratory cultures, more resistant sexual spores termed zygospores are formed by the union of hyphae of two different mold plants (Fig. 33). In this

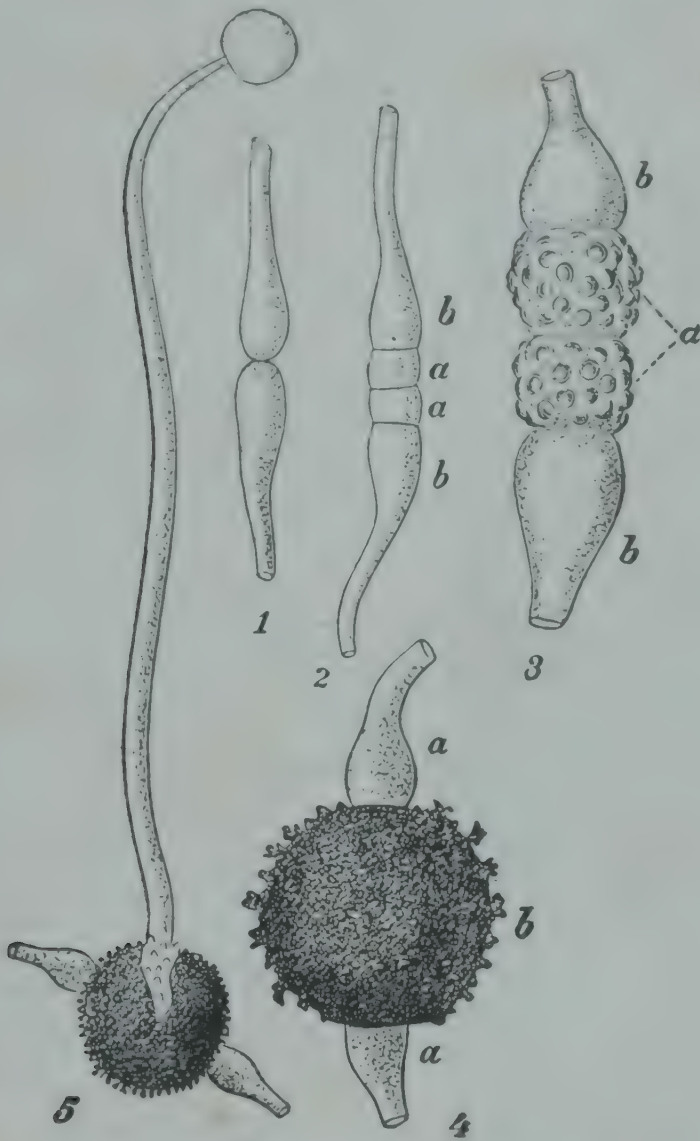


Fig. 33. Stages in zygospore formation in *Mucor mucedo* from terminal contact of two hyphae (1) to mature zygospore (4) and germination of the zygospore (5). (After Bredfeld. Reprinted from Skinner, Emmons, and Tsuchiya: *Henrici's Molds, Yeasts and Actinomyces*, John Wiley & Sons, Inc.)

case, the terminal cells of each hypha are sex cells or gametes separated from the rest of the hypha by a cell wall. The contacting gametes mature and eventually fuse to make a single cell, the zygospore, which develops a heavy, rough, black wall. Zygospores are resting spores which can lie dormant for a considerable period and then under suitable conditions can germinate the mycelium of a new plant.

*Mucor*. Several other common species of Phycomycetes belong to the genus *Mucor*. These nonseptate molds are widely distributed in the soil, on rotting

manure, fruits and other organic matter. The cottony growth grossly resembles that of *Rhizopus* (Fig. 34), but when viewed microscopically one discovers that *Mucor* possesses no stolons, the sporangiophores arise at any point on the much branched aerial mycelium and the columella is round (Fig. 35). The mode of sexual and asexual reproduction is similar to that of *Rhizopus*.

**Ascomycetes.** The Ascomycetes are a large and diverse group including some fleshy fungi, certain molds and the so-called true yeasts. The plant parasite *Claviceps purpurea* which attacks rye and other grains and produces the toxic substance ergot belongs here. In the past, particularly in Europe, the ingestion of such infected grains has resulted in ergot poisoning or ergotism in man and domestic animals. Among the Ascomycetes encountered in the home, in industry and in the laboratory, baker's yeast (*Saccharomyces cerevisiae*) and species of the mold *Penicillium* are common.

All molds belonging to this class have septate mycelia with aerial hyphae which arise singly from vegetative elements and which bear at their tips asexual spores known as **conidia**. Unlike the asexual spores of the Phycomycetes, conidia are not produced inside a sporangium, but are exogenous, free spores borne in chains at the tips of reproductive hyphae. As in the Phycomycetes, production of the asexual spores is the common mode of reproduction. The distinguishing feature of the Ascomycetes is the unique property of **ascospore** formation by all members of the group. Ascospores are sexual spores so named because they develop in an **ascus** or sac. Although a slightly different pattern is followed in different species, ascospore formation is usually initiated by the conjugation of gametes borne at the tips of two hyphae with fusion of their nuclei

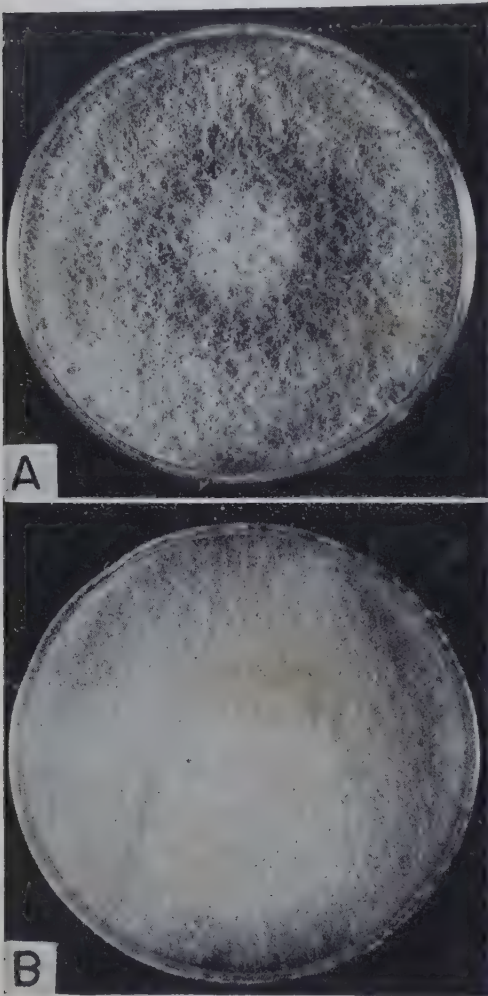


Fig. 34. *Mucor* (A) and *Rhizopus* (B) quickly fill the petri plate with a white to gray, cottony growth. (From Conant, et al.: *Manual of Clinical Mycology*, W. B. Saunders Co.)

(Fig. 36). In the common molds the resulting single cell germinates into the hyphae of a specialized mycelium. One binucleate cell near the tip of each of these hyphae becomes an ascus. The two nuclei of this cell fuse and by subsequent cell division form eight ascospores inside the ascus. Many asci thus become covered by a dense web or membrane, the **perithecium**, formed by the adjacent hyphae. Each ascospore can remain quiescent under adverse conditions



and can reproduce the mold plant when favorable circumstances prevail. Certain species of the common molds, such as *Penicillium*, form ascospores.

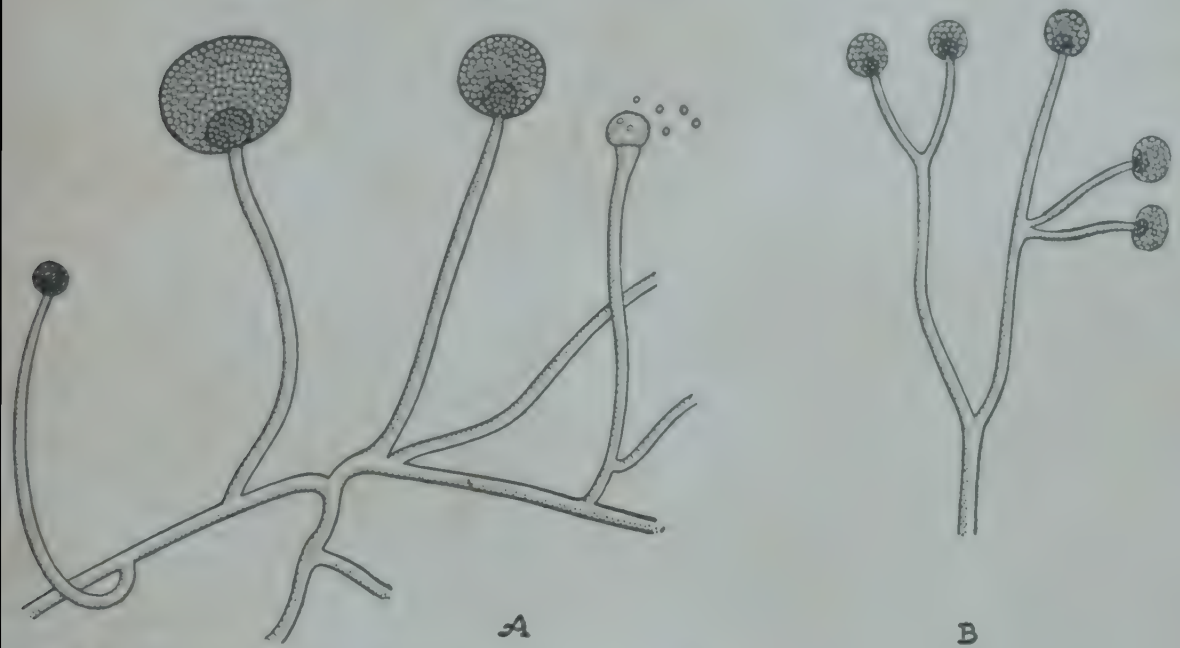


Fig. 35. Species of *Mucor* showing (A) unbranched sporangiophores and (B) branching sporangiophores. (Redrawn in part from Waksman and Starkey: *The Soil and the Microbe*, John Wiley & Sons, Inc.)

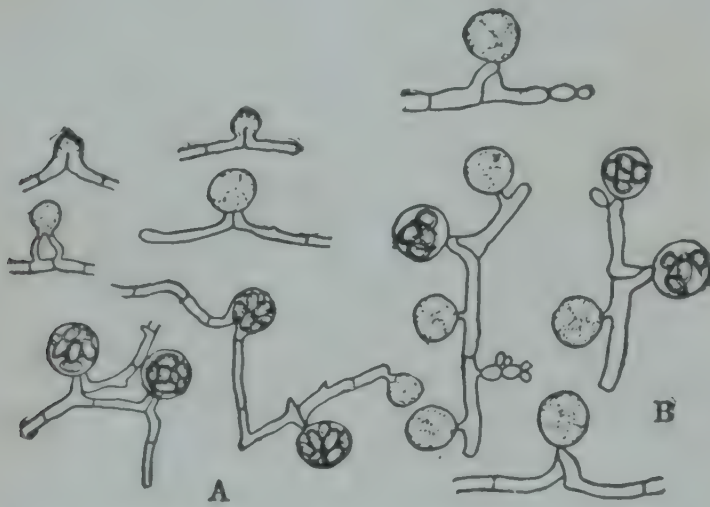
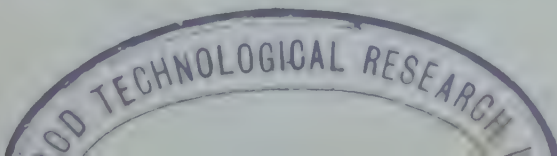


Fig. 36. Stages in the development of ascospores in two filamentous fungi. A. *Erenicascus fertilis*; B. *Endomycopsis fibuliger*. (After Guilliermond, in Skinner, Emmons and Tsuchiya: *Henrici's Molds, Yeasts and Actinomyces*, John Wiley & Sons, Inc.)

***Penicillium*.** The pigmented conidia of the penicillia, which give these molds their characteristic blue-green color, are small free cells arranged in chains at the ends of branched aerial hyphae (Fig. 37). The name *Penicillium*, derived from the Latin *penicillus* or brush, was suggested by the feathery, brush-like appearance of the conidiophores. The hyphae, both vegetative and reproductive, are septate. One of the common blue-green fruit molds is *Penicillium glaucum*. Other species



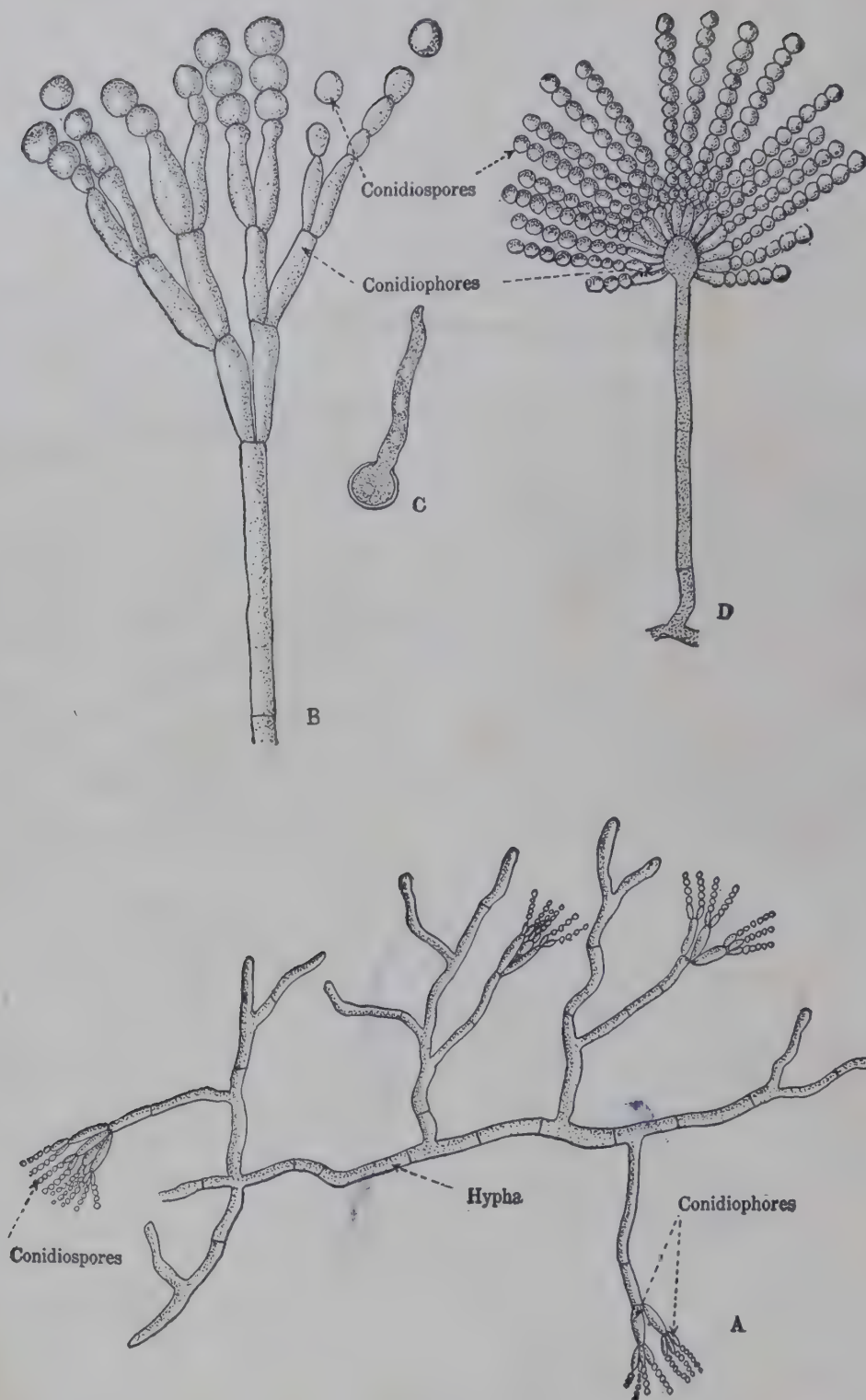


Fig. 37. Common molds. A. *Penicillium*; B. conidiophore of *Penicillium*; C, germinating conidiospore; D, conidiophore of *Aspergillus*. (From Holman and Robbins: *A Textbook of General Botany*, John Wiley & Sons, Inc.)

of industrial and medicinal value will be included in the discussion of the economic importance of molds.

**Fungi Imperfecti (Hyphomycetes).** Fungi which in so far as is known do not produce sexual spores are grouped together in the class Fungi Imperfecti



or, as it is also known, the class Hyphomycetes. Obviously this is a wastebasket group of otherwise unrelated fungi. Conidia and other kinds of asexual spores are formed and all the filamentous fungi of this group have a septate mycelium.

**Aspergillus.** These molds, frequently encountered as air contaminants, are distinguished by the round swollen tips of their conidiophores (Fig. 37). Chains of pigmented free spores arise like the spikes on an Indian club from all points of these globular structures until the tip of the mature conidiophore is completely concealed. The pigment in the conidia of different aspergilli varies. The most common species, *Aspergillus niger*, has sooty black conidia, while others appear

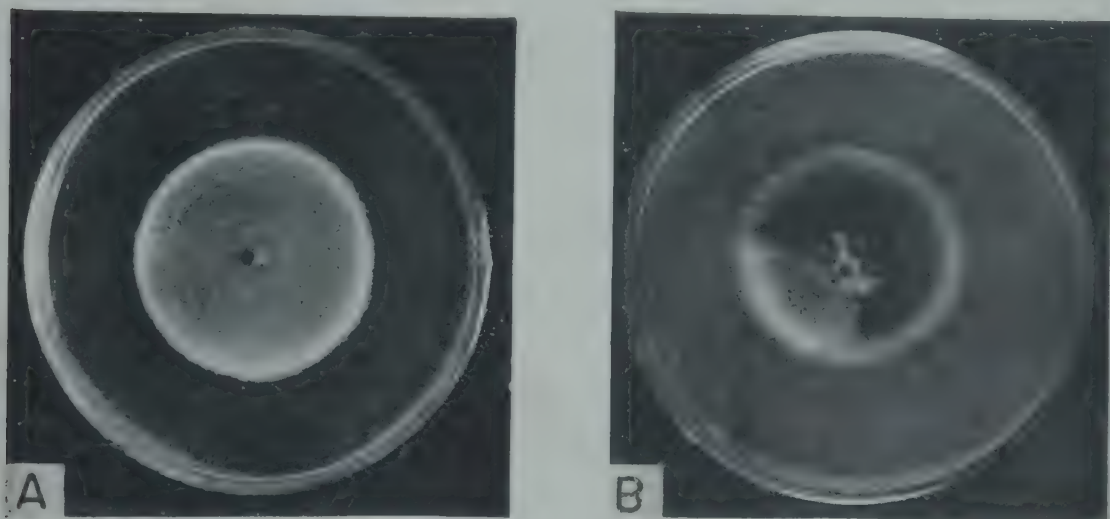


Fig. 38. A. *Penicillium* colony; B. *Alternaria* colony. (From Conant, et al.: *Manual of Clinical Mycology*, W. B. Saunders Co.)

golden to yellowish green. The ripe spore heads of *Aspergillus fumigatus*, the cause of a rare disease (aspergillosis) in birds and man, are bluish green. The vegetative mycelium of *Aspergillus* is septate, whereas the conidiophore is coenocytic. Certain species of aspergilli form sexual spores and these molds are classified as the genus *Eurotium* of the Ascomycetes.

**Alternaria.** The dark olive green or brown colonies of *Alternaria* (Fig. 38), a soil mold, are common air contaminants in laboratory cultures and are remarkable for the production of chains of large, multicellular, spindle-shaped spores termed **macroconidia** or **fuseaux** in contrast to the small, round, single-celled **microconidia** of many other septate molds. Several other genera of the imperfect fungi, some pathogenic for man, produce similar macroconidia. From time to time mycologists discover the sexual forms of some of these fungi and they are then transferred into the class Ascomycetes or in some cases into the class Basidiomycetes, depending on the kind of sexual spore produced. A wide variety of morphological types of fungi are classed in this group, including molds, yeasts and intermediate types.

**Yeast-like Molds.** Many of the Fungi Imperfecti occur at times as unicellular, yeast-like organisms and at others as mold-like, filamentous plants. To

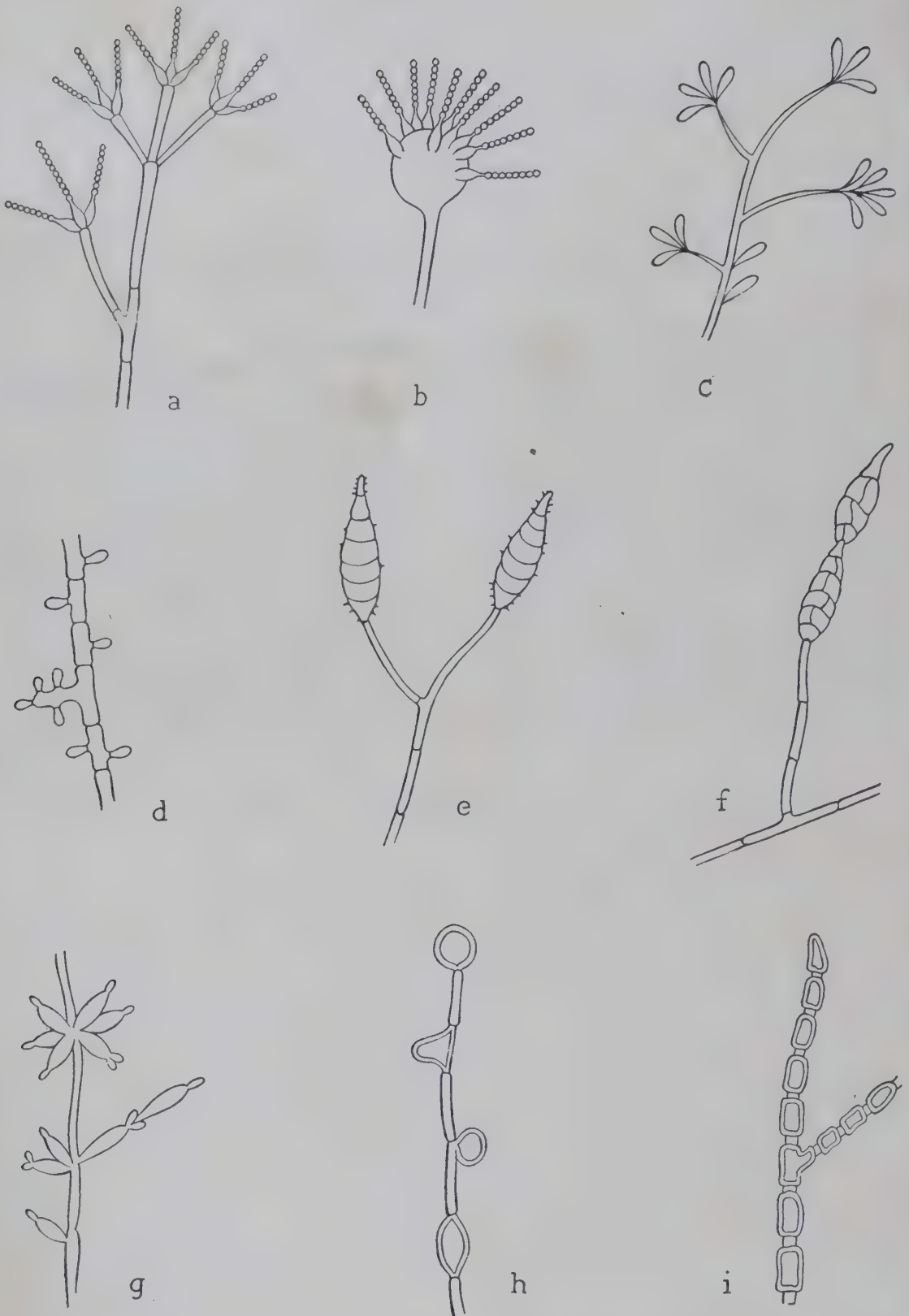


Fig. 39. Asexual spores of microscopic fungi. A–D, Microconidia; E and F, spindle-shaped macroconidia or fuseaux; G, blastospores; H, chlamydospores; I, arthrospores. See Fig. 32 for sporangiospores. (Redrawn from Conant, et al.: *Manual of Clinical Mycology*, W. B. Saunders Co.)



Understand the metamorphosis of such fungi it is advisable to consider the formation of free cells other than conidia by a variety of molds (Fig. 30). Under certain conditions, particularly when submerged in a liquid, the vegetative hyphae may form **arthrospores** by segmenting into thick-walled, rectangular, free cells. These cells, independent of the parent plant, can divide into other free, yeast-like cells or, if the recently divided cells remain attached, can produce a new mycelium.

In another type of vegetative reproduction, as seen in *Candida*, the septate mycelium does not fragment, but instead yeast-like cells or **blastospores** are given off as buds from the sides and ends of the hyphae. In many of these intermediate filamentous fungi the protoplasm in the mycelial cells becomes concentrated to form thick-walled, rounded spores having a diameter larger than that of the hyphae (Fig. 40). These resistant spores, termed **chlamydospores**, may or may not become separated from the mycelium.

Not only can certain yeasts germinate a mycelium and certain molds produce yeast-like organisms, but in some instances the same fungus may occur only as a yeast in one environment and only as a mold under different conditions. This is true of many of the pathogenic fungi. For example, members of the genus *Candida* are usually typical yeasts, unicellular, budding forms, when grown in aerobic laboratory cultures, but they may produce a rudimentary mycelium in anaerobic cultures and in the

issues of the host (Fig. 41). On the other hand, the important pathogens *Coccidioides immitis* and *Blastomyces dermatitidis* occur as yeast-like cells in the issues of the infected individual and as fluffy mold-like growths on certain laboratory culture media. Because of these morphological changes the classification of the pathogenic fungi is difficult and a problem for experts. The difficulty in recognizing the different forms of these fungi also explains why a variety of names has appeared in the literature for one and the same organism.



Fig. 40. *Candida albicans* from corn meal agar culture. Note filaments bearing chlamydospores. (From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Appleton-Century-Crofts, Inc.)

## YEASTS

As previously stated the yeasts are one-celled fungi that commonly reproduce by budding. They are regarded by some as degenerate eumycetes, fungi that have lost the ability to form a mycelium. Their relation to the other eumycetes is

based on the types of spores they produce. Under certain conditions the domesticated baker's and brewer's yeasts (*Saccharomyces*) form ascospores and are therefore, included in the Ascomycetes. Those that do not produce ascospores are usually placed in the class Fungi Imperfecti. The great majority of yeasts belong in these two groups, but there are a few which are classified with the Basidiomycetes. While most yeasts reproduce by budding, a few reproduce

asexually by transverse fission which divides the parent organism into two equal size, daughter cells.

**Structure of Yeast Cells.** Yeasts are oval, round or cigar-shaped cells averaging about  $5\ \mu$  in diameter and are considerably larger than bacteria. Each cell contains one distinct, usually excentric nucleus which may be seen by special staining technique (Fig. 42). A large portion of the mature yeast cell is taken up by a vacuole of clear fluid containing a single vibrating granule (Fig. 43). This granule activated by Brownian movement is termed the "dancing body" and is thought to be a particle of reserve food material, volutin, surrounded by a solution of the same material. The cytoplasm contains other inclusions such as granules of glycogen and volutin and small vacuoles of fat. Glycogen granules stain reddish brown with iodine, and the dissolved and particulate volutin may be colored pink by adding a dilute solu-

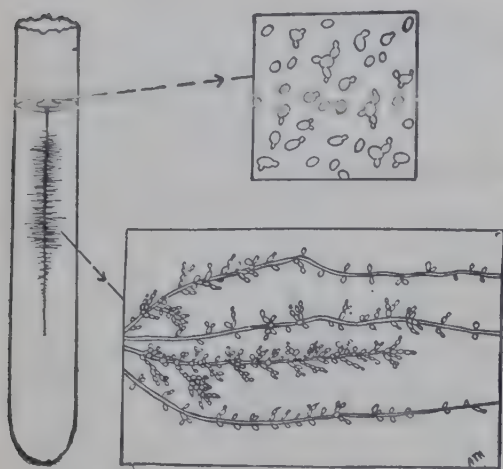


Fig. 41. Diagram illustrating the characteristics of the pathogenic *Candida*. In a gelatin stab culture only yeast-like cells are found at the surface, but filaments of mycelium radiate from the depths of the stab; these give rise to yeast-like cells by budding. (From Skinner, Emmons and Tsuchiya: *Henrici's Molds, Yeasts and Actinomyces*, John Wiley & Sons, Inc.)

tion of neutral red to the living cells. A thick rigid wall surrounds the protoplasm of the mature cell. During budding the cell wall weakens at one spot, the cytoplasm bulges out at that point, the nucleus divides and one daughter nucleus moves into the protuberance. As the bud enlarges, the wall constricts at its base and finally separates the young cell from the parent. If the two cells remain attached and budding of one or both occurs, a characteristic cluster or short chain is formed. In the fission yeasts the mature cell elongates and after nuclear division a cell wall is laid down across the center of the cell, dividing it into two equal-size cells.

**Ascospore Formation in Yeasts.** Some yeasts are known to produce ascospores by a sexual process, *i.e.*, by union of two cells. The two nuclei fuse and by repeated division of the zygote a fixed number of ascospores, usually four to eight, are formed. In *Saccharomyces*, the well known baker's and brewer's yeasts, fusion of cells is not observed, but the nucleus of a single cell divides, usually twice, to form four ascospores. Regardless of how the process is initiated,



At completion of the nuclear divisions the cytoplasm surrounding each nucleus separates from the rest of the protoplast, a spore wall is laid down around each mature ascospore and the original cell wall becomes the ascus (Fig. 43). The resulting spores can withstand environmental conditions which would destroy the vegetative yeasts. Ascospores are not seen in actively growing cultures. In fact, they are rarely formed in the laboratory unless young cultures are suddenly exposed to extremely adverse conditions such as complete desiccation or starvation. An environment favorable to growth stimulates the ascospore to germinate a vegetative yeast.

**Saccharomyces.** The common commercial yeast used in the baking and brewing industries and sold to the housewife in yeast cakes is *Saccharomyces cerevisiae*. As the genus name implies, these fungi are active sugar fermenters. They grow luxuriantly in solutions of sugar if only certain inorganic salts are also present, and their industrial value depends on their ability to ferment sugar

with the production of large quantities of ethyl alcohol and carbon dioxide. The cells are round or egg-shaped with a diameter of approximately 4 to 6  $\mu$ . They reproduce by budding and occasionally form ascospores. When a cell of *S. cerevisiae* multiplies on a solid medium, as on Sabouraud's agar, it gives rise to a

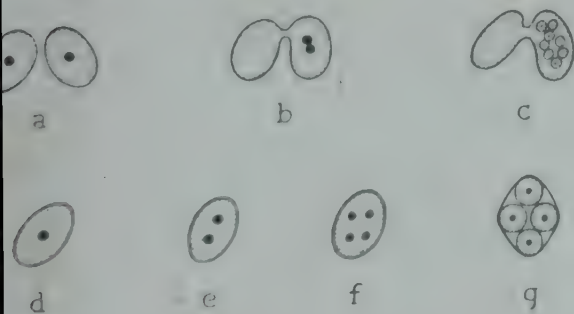


Fig. 43. Ascospore formation in yeasts. a, b, By conjugation; d-g, by parthenogenesis. (Redrawn from Henrici: *Biology of the Bacteria*, D. C. Heath & Co.)

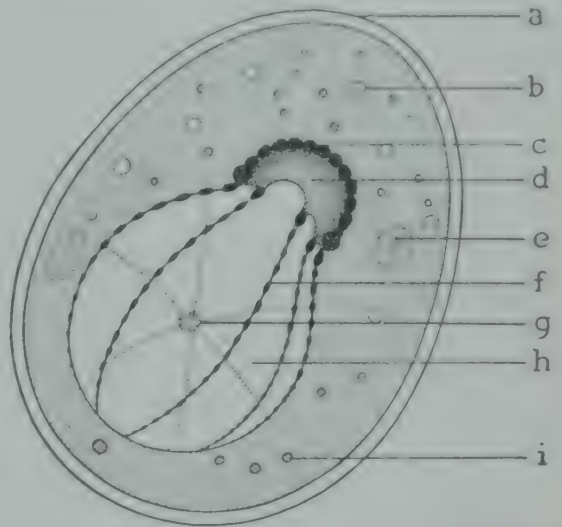


Fig. 42. Diagram of yeast cell. a, Cell wall; b, fat vacuole; c, d and f, nuclear structures; c, chromatin; d, nucleolus; e, glycogen vacuole; f, chromatin network; g, "dancing body" of volutin; h, nuclear vacuole; i, volutin granule. (Redrawn after Tanner: *Bacteriology*, John Wiley & Sons, Inc.)

round spot of white pasty growth about 1 to 2 mm. in diameter, the yeast colony. No mycelium is produced. Morphology of the cell and colony as well as fermentation reactions are used to identify this and other yeasts. *Saccharomyces cerevisiae* is not found in nature, but is now available only in the cultures man has handed down from generation to generation since antiquity. In biblical times a bit of dough or leaven was removed before each baking and

used as a starter for the next batch of bread. Leaven was really a crude culture in which there predominated a yeast with the desirable properties of producing a high yield of carbon dioxide and a good flavor. Today laboratories maintain

carefully selected strains in pure cultures. The ordinary yeast cake is a mass of living *S. cerevisiae* cells that have been removed from liquid cultures, mixed with a little flour or starch and compressed into solid form. A closely related

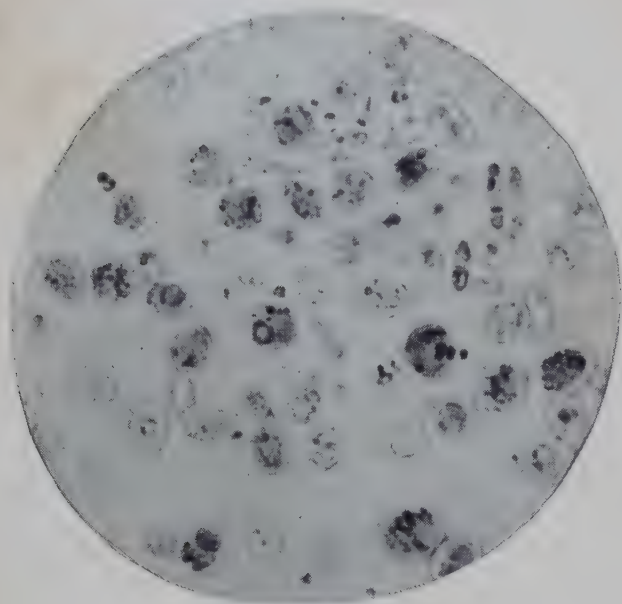


Fig. 44. *Saccharomyces cerevisiae* (Kral).

yeast, *S. ellipsoideus*, occurs in nature on fruits, especially on grapes, and in the soil of vineyards. When the juice of grapes or other fruits is allowed to undergo spontaneous alcoholic fermentation this and other wild yeasts are at work. Selected strains of *S. ellipsoideus* are employed in the wine industry.

**Asporogenous Yeasts.** The non-ascospore-forming yeasts and yeast-like fungi are classified with the Fungi Imperfecti. Included in this group are yeasts of the genus *Cryptococcus* whose budding cells form little or usually no mycelium. In the older literature the name *Torula* was often used for this genus. Some lactose-fermenting species of *Cryptococcus* are used, particularly in Europe, in the manufacture of fermented milk drinks, and they may also cause undesirable fermentations in cream and other dairy and brewery products.

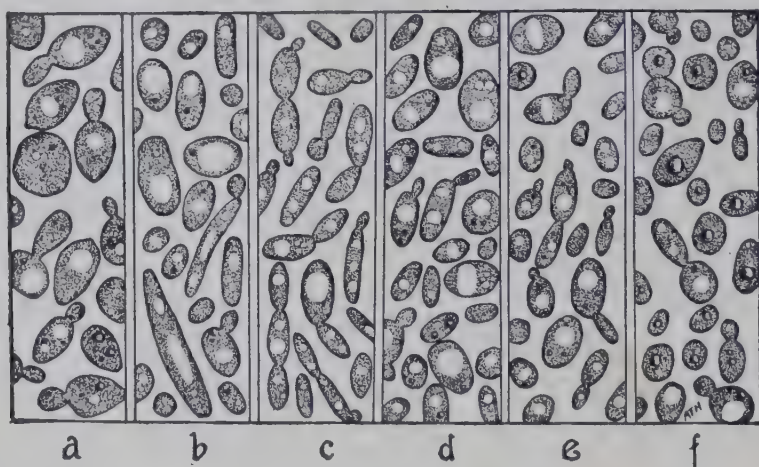


Fig. 45. Vegetative cells of some sporogenous yeasts (a, b and c) and asporogenous yeasts (d, e and f); d, "*Torula cremoris*"; e, *Rhodotorula glutinis*; f, *Cryptococcus pulcherrimus*. (From Skinner, Emmons and Tsuchiya: *Henrici's Molds, Yeasts and Actinomyces*, John Wiley & Sons.)

*Cryptococcus neoformans* (formerly known as *Torula histolytica*) is a nonfermenting pathogenic species that may cause a generalized infection in man and animals or, more commonly in the United States, a meningitis. Further discussion



of the disease **cryptococcosis** and its etiologic agent will be presented in Chapter 41.

Closely related to *Cryptococcus* are the film-forming yeasts of the genus *Mycoderma*. These along with certain ascospore-forming yeasts grow in a pellicle or scum on the surface of some wines and of sauerkraut, dill pickle and other pickle brines.

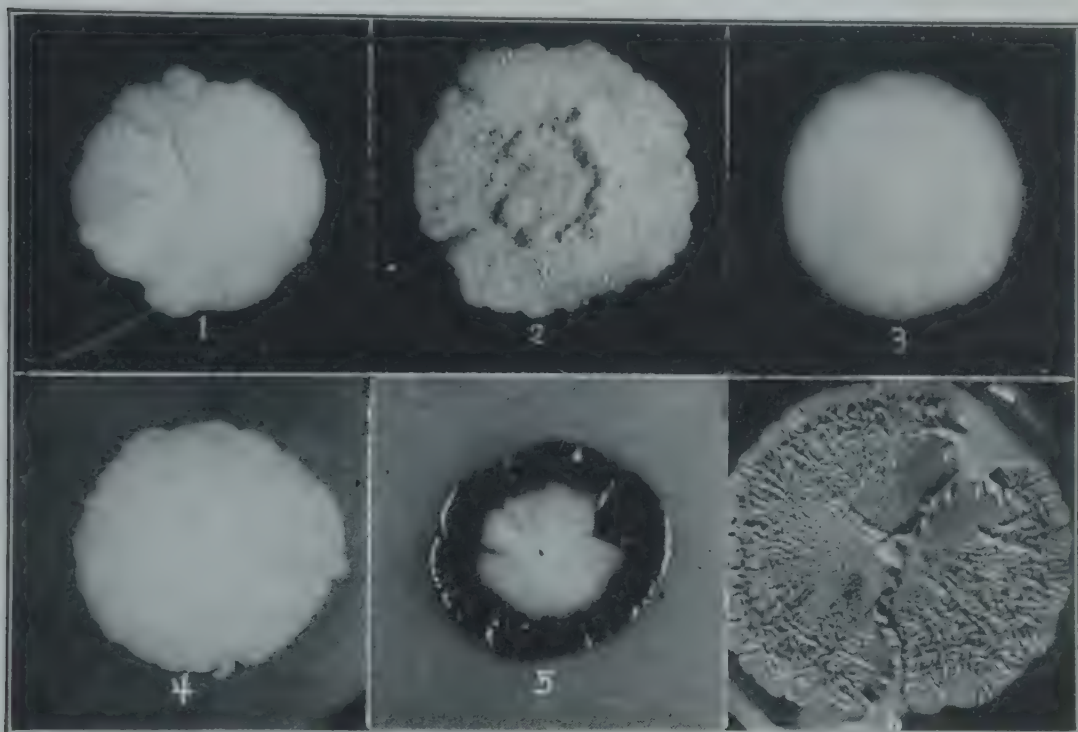


Fig. 46. Colonies of yeasts. 1, *Saccharomyces* sp. from soil; 2, *Pichia membranarum*; 3, *Hansenula anomala*; 4, "*Torula cremoris*"; 5, *Cryptococcus pulcherrimus*; 6, *Rhodotorula glutinis*. (From Skinner, Emmons and Tsuchiya: *Henrici's Molds, Yeasts and Actinomyces*, John Wiley & Sons, Inc.)

Members of the genus *Candida* (formerly known as *Monilia*) are predominantly yeast-like cells which under certain conditions produce a rudimentary mycelium. In young aerobic agar cultures these yeasts produce a creamy white surface growth of pasty consistency that is composed entirely of budding yeast cells. As the culture ages filaments often grow down into the medium. A mycelium also develops deep in agar stab cultures, apparently as a result of reduced oxygen tension. Microscopically the mycelium is seen to consist of simply branched, septate hyphae bearing yeast-like buds, the blastospores, on their sides and tips. Occasionally large, thick-walled chlamydospores may also be seen. Some species of *Candida* are important in certain brewing industries and in medicine. The parasitic "monilias," particularly *Candida albicans* (*Monilia albicans*), will be considered with the disease **moniliasis** (see page 463).

Black colonies of filamentous yeasts morphologically similar to *Candida* commonly grow on agar plates which have been exposed to the air. The relation-

ship of the "black yeasts" to the other eumycetes is not clear, and as a result they have a variety of names, including *Monilia niger* and *Torula niger*.

Other common air contaminants are yeasts of the genus *Rhódotorula*, whose coral to pink colored, soft, mucoid colonies are characteristic of the group. The oval budding cells seldom produce a mycelium. They differ from many other yeasts in that they do not ferment any of the sugars. They may, however, grow in acid and salt foods, such as sauerkraut and butter, causing discoloration of these products.

### PHYSIOLOGY OF YEASTS AND MOLDS

**Nutrition and Respiration.** Various kinds of yeasts and molds differ in their nutritional requirements, but all need organic carbon which may be supplied by such substances as sugars, organic acids, aldehydes and glycerol. Products of protein decomposition, particularly amino acids and ammonia, may serve as sources of nitrogen, and in addition certain molds can utilize the nitrogen of nitrate salts. Water, mineral salts and accessory growth factors or vitamins are also required.

The majority of fungi can digest and use a variety of carbohydrates, proteins and, to a limited extent, fats, and in this respect their nutrition resembles that of animals. One great difference between the nutrition of animals and fungi, however, is that digestion in animals is performed inside the body and in the case of the fungi it is an external process. The vegetative mycelium of a mold secretes digestive enzymes which may diffuse for considerable distances from their source. Seeing a mold growing on a substrate such as syrup, fruit, bread, cheese, wood or leather is proof that it has found suitable nutrients in solution or that it has the proper digestive enzymes to change materials in the substrate into a solution of adequate nutrients. Molds can readily attack starches and even more complex polysaccharides such as the cellulose of green plants, surpassing in these amylolytic activities both the yeasts and the bacteria. Molds are also well equipped with proteolytic enzymes which decompose many kinds of proteins. Fats are less readily digested although spoilage of fat foods, such as butter, is common. There are few organic materials that cannot be decomposed by some kind of mold. Digested foods are absorbed and used as they are in other organisms, as a source of energy and in the synthesis of protoplasm.

Sugars are acted on by the respiratory enzymes inside the mold cells which partially oxidize or ferment them chiefly to organic acids, including citric, oxalic, gluconic and fumaric acid, with concomitant release of energy to the organism. The acids diffuse from the plant as waste products or the incomplete utilization of the sugars may in many molds progress to complete oxidation whereby the acids are broken down into carbon dioxide and water with further liberation of energy. Most molds must have atmospheric oxygen to accomplish these changes and they are, therefore, strictly aerobic.

Yeasts, like molds, secrete digestive enzymes into their surroundings and



absorb the digested nutrients. However the entire yeast plant, being unicellular, is engaged in these activities. While not as highly proteolytic as the molds and not equipped with amylolytic enzymes for the digestion of starch and other polysaccharides, the baker's and brewer's yeasts are vigorous fermenters of the sugars glucose, maltose and sucrose. Disaccharides such as maltose and sucrose and other sugars more complex than the monosaccharides must first be digested to the simple sugar glucose before fermentation begins. A system of respiratory enzymes in the yeast cell then splits the glucose molecule by a complicated series of reactions into ethyl alcohol and carbon dioxide. The summary equation of these reactions follows:



This incomplete oxidation or fermentation of sugar by which energy is liberated to the cell proceeds in the absence of atmospheric oxygen. Yeasts can grow in air or without air, but aerobic cultivation diminishes the production of alcohol and results in a high yield of carbon dioxide.

**Cultivation of the Yeasts and Molds.** To isolate yeasts and molds from their natural habitats it is necessary to provide conditions which encourage their development and suppress that of the more rapidly growing bacteria. Molds and yeasts differ from most bacteria in their ability to tolerate relatively high acidity (low *pH*) and osmotic pressures created by high concentrations of sugar and salt as evidenced by their growth on syrups, jellies, salted foods and in pickling prunes. Laboratory media of high sugar content and high acidity are used to this end, for a *pH* around 5 to 6 inhibits most bacteria but is preferred by yeasts and molds, while the sugar-enriched substrate stimulates their development. Henrici's dextrose tartaric agar and Sabouraud's sugar agar are excellent media for this purpose. (See Appendix for formulae.) Infusions of corn meal, prunes or potatoes often enriched with glucose are also used. Wort agar made from a base of fermenting malt infusion is particularly suited to the cultivation of yeasts. Many fungi will grow on synthetic media in which nitrogen is supplied in the form of inorganic salts, ammonium or nitrate compounds, and a sugar provides the organic carbon. A medium recently developed for the primary isolation of pathogenic fungi is Littman's oxgall agar, a dextrose agar which depends on crystal violet and streptomycin, rather than a low *pH*, to inhibit the growth of bacteria; oxgall restricts the spreading of the fungus colonies.

Once isolated, pure cultures of eumycetes may be maintained and studied in liquid as well as solid media. This is especially advantageous in the study of yeasts which are identified according to their fermentation reactions. Furthermore, different kinds of yeasts grow characteristically either as a sediment at the bottom of liquids or as a surface scum, and in the brewing industry these are referred to as "bottom yeasts" and "top yeasts" respectively.

Most of the free-living fungi have an optimum temperature around 25° to 30° C. although different species vary in this respect. For example, the blue-green

molds such as *Penicillium* grow best at 20° to 24° C., while the aspergilli prefer 35° to 40° C. The best growth temperature for many yeasts is approximately 24° C. or room temperature.

### RELATION OF YEASTS AND MOLDS TO HUMAN WELFARE

The activities of the microscopic eumycetes as they influence human welfare may be considered from three standpoints: those helpful to man, those acting as nuisances or involving economic loss and those causing human disease. Man is dependent on the fungi for the part they play in soil fertility, in the preparation of certain foods, in the brewing industry and in many other industries based on fermentations. On the other hand, human efforts are thwarted and an immense money loss is suffered annually from crop failures due to fungus diseases of plants and from food spoilage as well as the disintegration of other materials by yeasts and molds. Relatively few species parasitize man, yet these few are a constant threat and the cause of some important human infections.

**The Food Cycle in Nature.** Soil fertility and therefore the production of food for man and animals depends largely on the biochemical activities of the microorganisms living in the soil. Nutrition of the saprophytic fungi involves primarily the decomposition of complex organic matter, actually the dissolution of dead plant and animal bodies as well as the waste products of organisms. The enzymes of yeasts and molds along with those of the bacteria living in the soil transform plant and animal carbohydrates, proteins and fats into simple inorganic substances, mineral salts such as nitrates, phosphates and carbonates which are essential to the growth of green plants. Without this action the soil would become barren; no green plants and eventually no animals could survive. This interdependence of organisms for food constitutes a chain of events known as the **food cycle** in nature. Molds, being better equipped with amylolytic enzymes than yeasts or most bacteria, are responsible for much of the aerobic decomposition of plant bodies.

**Manufacture of Foods.** So important is baker's yeast as a leavening agent that the production of compressed yeast cakes and powdered yeasts is an industry in itself. Successful bread making depends on providing the proper conditions for growth of the yeast cells. *Saccharomyces cerevisiae* cannot use the starch of the flour, but the sugar in the dough supplies an available carbohydrate which the yeast ferments to alcohol and carbon dioxide. The latter caught as gas bubbles in the dough causes it to rise. During the baking process the alcohol is dissipated by heat and the carbon dioxide expands to give a light, porous texture to the product.

*Saccharomyces cerevisiae* has been found to be a valuable source of vitamins, especially of thiamine (B<sub>1</sub>) and riboflavin (B<sub>2</sub>). Irradiated yeast is rich in vitamin D and such yeast fed to cows increases the content of this vitamin in the milk.

Recent investigations have shown that yeasts may furnish a huge, hitherto untapped food supply which might be expedient in times of famine and war when



shortages of proteins and fats invariably threaten health and survival. Growing solely on sugar and common mineral salts, yeasts can multiply to an enormous bulk in a short time. The dried cells are over 50 per cent protein and in some species a high percentage of fat is synthesized.

Camembert, roquefort and the blue cheeses owe their flavors to the enzymes of certain molds acting on the curd during the ripening process. Thus in the manufacture of camembert cheese, *Penicillium camembertii* is allowed to grow on the surface of the soft curd, its enzymes penetrate the curd and the resulting proteolysis imparts the characteristic taste and texture. *Penicillium roquefortii* is introduced into and grows throughout the relatively dry, salty curd of roquefort cheese.

**Industrial Fermentations by Yeasts.** Certain strains of *S. cerevisiae* are employed by brewers in the production of the malt beverages, beer and ale. The yeasts ferment the maltose in a mash which has been made from sprouting barley seeds to form alcohol and carbon dioxide. The difference in the flavor of beer and ale is due to different ingredients or quantities of ingredients, particularly in the amount of hops added to the mash, and to different strains of yeast. Surface-growing "top yeasts" produce ale and "bottom yeasts" operate in the depths of the beer vat.

Wines are the result of the fermentative action of yeasts, usually *S. ellipsoideus*, on the sugar in fruit juices. Selected strains are maintained and used as starters and, as in the brewing industry, every step in the production is protected against contaminations. In the spontaneous fermentations occurring in home-made wines and cider the yeasts together with other microorganisms are introduced on the skins of the fruit. Distillation of yeast-fermented, carbohydrate mashes and juices produces beverages of higher alcohol content, spirits such as whiskey, rum and brandy.

Yeasts are also employed in the production of commercial alcohol. Cheap carbohydrate materials, crude molasses or sugar solutions made by the hydrolysis of the starch and cellulose from corn-cobs and sawdust, are fermented to manufacture the alcohol used in industrial solvents, drying agents and the like.

Glycerin may be produced by a modification of ordinary yeast fermentation of sugar. Of tremendous economic importance, this substance is useful as a solvent, an anti-freeze, a sweetening agent, in the manufacture of explosives, in medicinals, antiseptics, adhesives and inks.

**Commercial and Medicinal Uses of Molds.** The amylolytic enzymes of molds, diastase for example, are put to work in some industries where the conversion of starch to sugar is required as a preliminary step in the production of commercial alcohol and in the manufacture of adhesives and sizings. One preparation of amylolytic mold enzymes is sold under the trade name "taka-diastase." Among the mold enzymes serving man are those which yield citric acid and gluconic acid from sugars in quantities large enough to be of commercial value. Both these products are incorporated, as citrates and gluconates respectively, in certain drugs, and citric acid is also widely used as a flavoring agent. Gallic

acid, important in the dye and ink industries, is produced from tannin by mold enzymes. The proteolytic enzymes of some molds play an important role in the manufacture of glue and in the tanning industry. The mold *Claviceps purpurea* is the source of the drug ergot and its derivatives which are used in medicine. The discovery that the mold *Penicillium notatum* produces penicillin, a substance which is antagonistic to certain pathogenic bacteria, introduced a new type of therapy for infectious diseases and stimulated the search for other antibiotic substances. A number of molds are now recognized as sources of a variety of such substances (see page 210).

**Economic Loss Due to Yeasts and Molds.** Foods which will not readily support the growth of bacteria are often subject to spoilage by yeasts and molds. Such foods often have too little moisture, too high a sugar or salt content, or are too acid to serve as culture media for bacteria. Yeasts frequently cause undesirable fermentations in jellies, syrups, condensed milk and preserved fruits. Molds attack these sweet foods even more commonly than yeasts, as well as pickled foods, butter, cheese, preserved meats and relatively dry foods such as bread and stored fruits and vegetables. It is not uncommon to find molds growing on foods that have been refrigerated for a long time. Damp fabrics mildew, the leather of shoes, harness and book bindings may become moldy and rot, and even paper may be destroyed by these fungi. The eumycetes parasitize green plants much more commonly than they do animals and man. Probably every species of plant is susceptible to at least a half-dozen different parasitic fungi including molds, mildews, rusts and smuts. The economic loss incurred by mycotic diseases of grain and fruit crops is enormous.

**The Pathogenic Fungi.** While most infectious diseases of man are of bacterial or viral etiology, infections caused by yeasts and molds are by no means uncommon and some of them are among the most serious of human afflictions. Pathogenic fungi include morphological types which run the gamut from typical molds through the yeast-like molds of the Fungi Imperfecti to permanently one-celled yeasts. Ringworm of the skin, scalp and nails as well as the important mycotic infections of the mucous membranes and deep tissues of the body will be discussed later in Chapter 41.



# 6

## THE BACTERIA

**Nature of the Bacteria.** The bacteria are a group of one-celled organisms that lack chlorophyll and reproduce by binary fission. They are smaller than the other unicellular fungi although the size of the largest bacteria approaches that of the smallest yeasts. Certain bacteria may occasionally multiply by other methods, but reproduction by binary fission is the rule among the majority. They are, therefore, designated as the fission fungi and are classified in a separate class, the *Schizomycetes*, to distinguish them from the fungi which usually multiply by spore formation or budding.

No single characteristic of the bacteria differentiates them from other microorganisms, but the combination of their size, cell structure, lack of chlorophyll and method of reproduction is distinctive. The absence of a mouth or any mechanism for the ingestion of solid food, the possession of a rather rigid cell wall and certain cell constituents serve to separate the bacteria from the protozoa and indicate their relationship to the plant kingdom. Their lack of chlorophyll places them with the fungi, but certain other characteristics of the bacteria do not coincide with those of the typical colorless plant. Their position in the plant kingdom may be justified simply by the fact that they are more like plants than like animals. The question of the bacterial nucleus, which will be discussed more adequately later, is also significant in distinguishing bacteria from other chlorophyll-free microorganisms. Suffice it to say that a nucleus *similar* to that found in the cells of other organisms has never been demonstrated in cells of bacteria or of the blue-green algae, although nucleoproteins characteristic of the nucleus of higher organisms are present in the bacterial cells.

**Orders of Bacteria.** Actually there is wide morphological variation within the Schizomycetes, and the range of types extends from distinctly mold-like to protozoan-like bacteria. It is practical to separate the entire class of fission fungi into the "true bacteria" or order **Eubacteriales** and several orders of the so-called "higher bacteria" which include those listed below.

### EUBACTERIALES.

The true bacteria. The great majority of common bacteria, including:

- (1) spherical shaped bacteria or **cocci** (sing., **coccus**),
- (2) rod-shaped bacteria or **bacilli** (sing., **bacillus**), and
- (3) curved to spiral rod-shaped bacteria or **spirilla** (sing., **spirillum**).

## ACTINOMYCETALES.

The **branching, mold-like** bacteria including short, rod-shaped and filamentous forms. Certain filamentous members may produce conidia.

## CHLAMYDOBACTERIALES.

Bacteria enclosed in filamentous **sheaths**. The bacteria within the sheaths may resemble the true bacteria or they may be filamentous. Included here are the "iron bacteria" whose sheaths contain inorganic iron compounds.

## MYXOBACTERIALES.

The **slime** bacteria whose life cycle consists of a swarm stage and a resting stage. In the swarm stage the rod-shaped cells of the colony secrete and move in unison through a mass of slime. In the resting stage, movement ceases and the cells of the colony give rise to fruiting bodies, reproductive spores or cysts.

## SPIROCHAETALES.

**Protozoan-like** bacteria. The cells are slender flexuous spirals.

Only certain members of the Eubacteriales, Actinomycetales and Spirochaetales are of medical importance.

## THE EUBACTERIALES

The true bacteria are small undifferentiated organisms of three morphological types: spherical cells or cocci, straight rod-shaped cells or bacilli and curved rods or spirilla. Normally none of these bacteria branch. They retain their shape by virtue of a relatively rigid cell wall. The size of these bacterial cells differs with their age and the environmental conditions. As a rule, bacteria in young cultures are larger than those in older cultures of the same species grown under the same conditions. Another factor leading to differences in measurements is the type of microscopic preparation in which the bacteria are observed. Living cells are larger than those seen in most dried, fixed mounts. Nevertheless, the average size of the chief morphological types of bacteria can be stated approximately. Bacteria and other microscopic bodies are measured in terms of **microns**. A micron (represented by the Greek letter mu,  $\mu$ ) equals  $1/1000$  of a millimeter or  $1/25,000$  of an inch. The diameter of the average coccus is about  $1 \mu$ . Small rod-shaped bacteria are usually around  $0.5 \mu$  in width by  $1.5 \mu$  in length, while a bacillus is considered large if it measures  $1 \mu$  by 3 or  $4 \mu$ . The size of different bacilli and spirilla varies considerably, the cells of certain species attaining a length of 10 to  $15 \mu$ . In order to gain some notion of their actual size, one must realize that the bacteria are magnified almost one thousand times in the usual stained microscopic preparation as observed through the oil immersion objective and the  $10\times$  ocular of the microscope.

Some species of the Eubacteriales are motile and one or more bacterial flagella can be demonstrated on these organisms. Certain rod-shaped cells may enter into a resting stage by becoming resistant spores. Reproduction is by binary fission and characteristic cell groups are formed.



The true bacteria are undoubtedly the most ubiquitous of living organisms. They have been brought back from Little America in the Antarctic, from the stratosphere and from the depths of the ocean. They abound in all natural waters, in hot springs, snow, ice, soil, air, on the external surfaces of plants and in all parts of the animal body that come in contact with the external environment. The internal tissues of plants and animals are, however, normally free from bacteria. In other words, bacteria have become adapted to almost every kind of environment, although not all bacteria are able to live in all environments. Certain kinds occur or are more prevalent in one place than another depending upon the environmental circumstances and their ability to adapt themselves to those circumstances. For example, spirilla are common in water, a high proportion of soil bacteria are bacilli and many varieties of cocci inhabit the animal body.

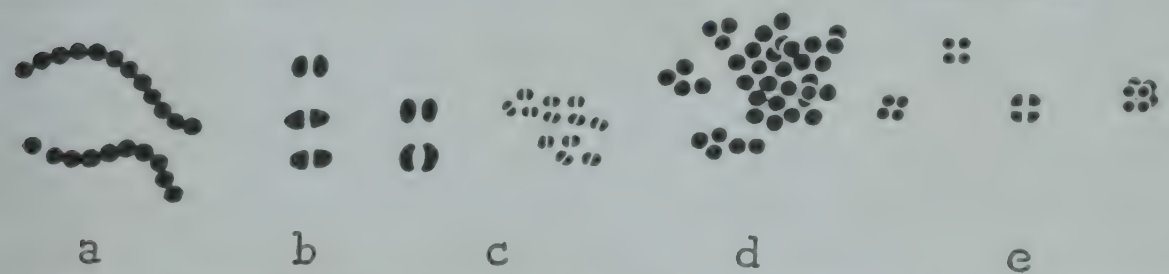


Fig. 47. Common morphologic types of cocci. a, *Streptococcus*; b and c, diplococci including (b) gram-positive cocci of the genus *Diplococcus* and (c) gram-negative cocci of the genus *Neisseria*; d, *Staphylococcus*; e, *Sarcina*.

**The Cocci.** Bacteria are three-dimensional bodies: the bacilli are actually cylinders with thickness as well as width and length and the cocci are commonly spherical, like marbles. The line of division in many cocci remains marked by their flattened or indented inner surfaces. For this reason certain cocci are said to be biscuit- or bean-shaped. Some cocci become conspicuously elongated or oval in shape before dividing into two equal-size daughter cells.

The cocci that have originated from the same parent cell tend to grow in microscopic groups of definite arrangement. Two factors are of prime importance in determining this arrangement of the cells: the successive planes through which the cells are split during fission, and whether the recently divided cells remain together or separate after fission. When a single coccus divides, the flattened inner surfaces of the two new cells represent the first plane of fission. These two cocci generally remain together, and when mature may give rise to one of several cell arrangements. If the two cells next divide in such a way that the plane of fission in each is the same as that of the first division then a chain of four cocci will result. These four cells will either remain attached to each other in the chain, or they will separate into two pairs of cocci. Cocci which thus divide by successive parallel planes and remain together in long or short chains are named **streptococci** (sing., *streptococcus*), while those which occur regularly in pairs are known as **diplococci** (sing., *diplococcus*). If different planes of fission are involved in successive cell divisions, flat plates or three dimensional clusters of

cells are formed. These cell groups may be irregular clusters resembling bunches of grapes or they may be regular tetrads (four-celled groups) or cubical packets of eight cells each. The parasitic cocci that occur in irregular clusters are known as **staphylococci**, whereas many common saprophytic cocci that are grouped in flat plates or irregular masses are termed **micrococci**. The generic names *Micrococcus* and *Staphylococcus* are often confused, for there is no sharp distinction between these bacteria on the basis of their microscopic appearance, and recently it has been suggested that they be merged into the single genus *Micrococcus* (Bergey, 1948). A parasitic coccus that characteristically gives rise to tetrad groups was once considered a *Micrococcus*, but recently such cocci have been assigned a new generic name, *Gaffkya*. Cocci of the genus *Sarcina* are arranged in regular cubical packets formed by successive divisions in three planes. Thus a single sarcina divides to form a pair, the next division which occurs at right angles to the first results in a tetrad, and these four cells cut through by the third plane of fission become a cube of eight cells. These three planes of fission repeated in sequence can form cell groups of sixteen, thirty-two and so on until other factors limit the size of the packets.

Since each kind of bacterium has a characteristic cell arrangement, this trait offers an important clue in their identification. In the case of the cocci it is the primary basis of their classification and it determines their generic name. Paired cocci are separated into two genera according to their positive or negative reaction when stained by Gram's method, a technique fully described under microscopic methods (Chapter 9). A summary description of the common and important genera of the cocci is given below. It should be noted that whereas the term diplococcus means any paired coccus, the same word begun with a capital letter refers only to the gram-positive paired cocci of the genus *Diplococcus*.

GENUS	MICROSCOPIC APPEARANCE
<i>Streptococcus</i>	Cocci occurring singly, in pairs and in <b>chains</b> .
<i>Diplococcus</i>	Gram-positive cocci, singly and in <b>pairs</b> .
<i>Neisseria</i>	Gram-negative cocci, singly and in <b>pairs</b> .
<i>Staphylococcus</i>	Cocci, singly, pairs and <b>irregular clusters</b> .
<i>Sarcina</i>	Cocci, singly, pairs, tetrads and <b>regular cubical packets</b> .

With the exception of the *Neisseria*, cocci are generally gram-positive. They are further characterized by their inability to form endospores, resistant bodies which will be more adequately described later, and by their lack of motility.

**The Bacilli.** The bacilli are rod-shaped bacteria whose form varies according to the character of their ends and the relation of the length of each cell to its width. The ends of bacilli may be rounded, pointed or truncated, *i.e.*, square cut or flattened. There is no difficulty in identifying a bacterium as a bacillus if its length is two or three times its width, but in the case of short plump rods with rounded ends, cells really oval in shape, the decision as to whether the cell is a bacillus or a coccus is sometimes a difficult one to make. This is especially a problem for beginning students who are confused by the newly divided forms of



short rods and the pre-fission, elongated forms of cocci. Although there is no sure rule to guide the novice, it is helpful to keep in mind that in an isolated colony or in a pure culture of an actively growing bacterium, young, post-fission cells are only half the size of the mature forms, and that if one axis of the mature cells is distinctly longer than the other in most cases the organism is a bacillus.

The bacilli always divide in a line that cuts across the short axis of the cell at right angles to its length. It follows that rod-shaped bacteria may occur only in three different cell arrangements, singly, in pairs and, according to the tenacity with which the cells remain attached after fission, in long or short chains. It is characteristic for some species to form long chains and for others to develop

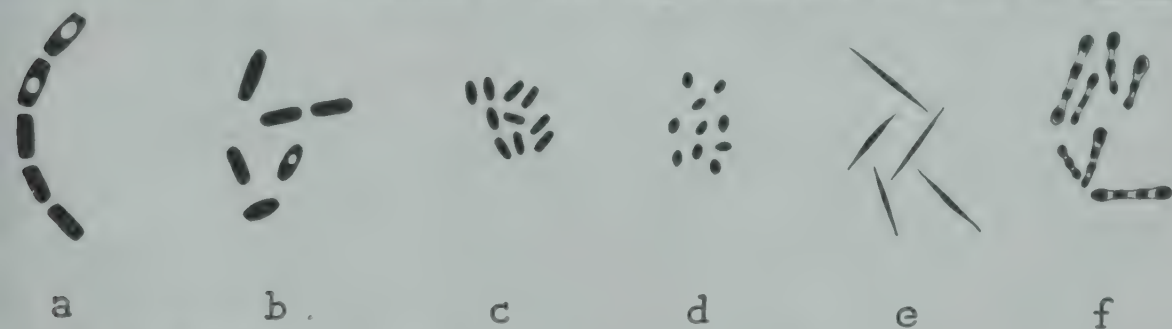


Fig. 48. Morphologic types of bacilli. a, Chains of large, spore-forming bacilli which have truncate ends; b, c and d, size differences in common rod-shaped forms characterized by rounded ends and cell groups occurring singly and in pairs; b, a spore-forming bacillus; c, bacilli showing slipping type of post-fission movement; d, small, ovoid forms known as coccobacilli; e, rods with tapering or pointed ends, fusiform bacilli; f, granular staining bacilli showing snapping type of post-fission movement.

only an occasional short chain of three or four cells. The microscopic pattern assumed by bacilli is determined also by certain movements exhibited when the connection between two sister cells is finally severed. These **postfission movements** are (1) **slipping**, which causes one cell to slip back and lie parallel to the other, (2) **whipping**, by which one cell moves around the other until an angle like an L or a V is formed or until the two cells lie parallel, and (3) **snapping**, whereby the cells trying to separate bend, break apart and, finally, lie across each other as may happen when a twig is bent and then snapped in two. Postfission movements are responsible for parallel or palisade arrangements, for angular, zig-zag configurations and for bends and breaks in chains of bacilli.

Although each bacillus has its characteristic cell grouping, this feature is not important in the classification and naming of the rods as it is in the case of the cocci. It is rare for any bacteria except certain bacilli to produce endospores. Once the rod shape of the cell has been ascertained, the next fact to discover when trying to identify a bacillus is, therefore, whether or not it forms endospores. All rod-shaped bacteria fall into two great categories: the endospore-forming and the nonsporulating rods. But information gained by microscopic observation alone is not sufficient to establish the genus of a rod-shaped bacterium. In the case of the spore-forming rods, their relation to atmospheric oxygen must be learned. If they are aerobic sporulating bacilli, their genus name is *Bacillus*. If they will

not grow in the presence of air or atmospheric oxygen, that is, if they are anaerobic sporulating rods, they belong to the genus *Clostridium*. It should be noted that the word bacillus begun with a small letter is used to indicate any rod-shaped bacterium, while the same word begun with a capital letter is a generic name used to designate one kind of rod, namely the sporulating aerobes. The classification and identification of the nonsporulating rods depend on a wide variety of physiological properties. One must learn, chiefly by cultural and chemical tests, what they do before determining their identity. That there are over forty genera of nonsporulating rods is evidence of the great diversity among this group. The spore-forming bacilli are usually gram-positive, whereas the majority of nonsporulating rods are gram-negative. Another ge-

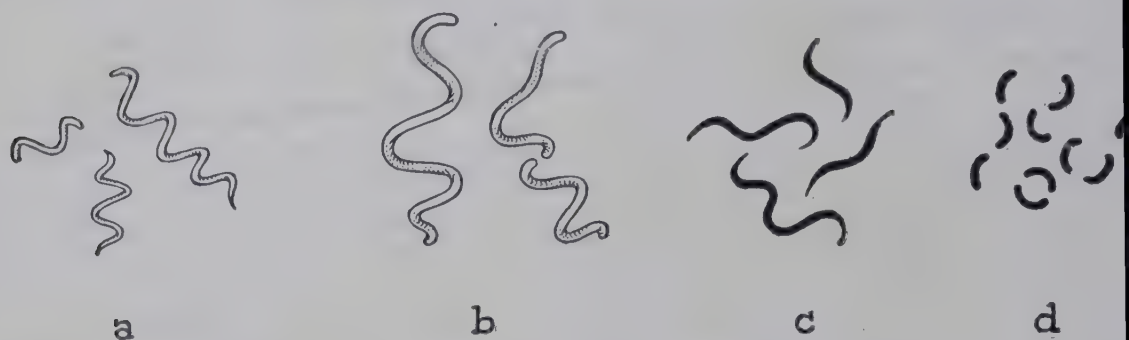


Fig. 49. Types of spirilla. a, b and c, *Spirillum*; d, *Vibrio*.

eralization which holds true in most cases is that the average spore-forming rod is larger than a nonsporulating bacillus. There are motile and nonmotile aerobic and anaerobic species among both kinds of rod-shaped bacteria. Endospore formation, which will be described later, is not a method of reproduction.

**The Spirilla.** The spirilla are rigid bent rods, some of which are short with but a single curve like a comma or parenthesis sign, while others are long, curved or spiral cells. Bacteria of this shape are subdivided and named according to the length of their cells and the number of curves they possess. Short bent rods generally having a single terminal flagellum are given the generic name *Vibrio*. Longer, more spiral bacteria possessing a tuft of several polar flagella are designated as the genus *Spirillum*. The word spirillum is used, then, to indicate either a general morphological type, the curved rod, or the genus *Spirillum*, depending on whether it is capitalized or not. Certain curved rods have peculiar metabolic properties and are classified accordingly. For example, separate genera have been established for the cellulose-oxidizing vibrios (*Cellvibrio*; *Cellfalcicula*), the anaerobic vibrios which reduce sulfate to hydrogen sulfide (*Desulfovibrio*), the spiral sulfur bacteria (*Thiospira*; *Thiospirillum*), and photosynthetic spirilla of the genus *Rhodospirillum*. Since the spirilla usually separate soon after fission, they occur singly, in pairs and in short, spiral chains. These bacteria never form endospores; they are gram-negative and almost always motile. Reproduction, as in the straight rods, is by transverse, binary fission.



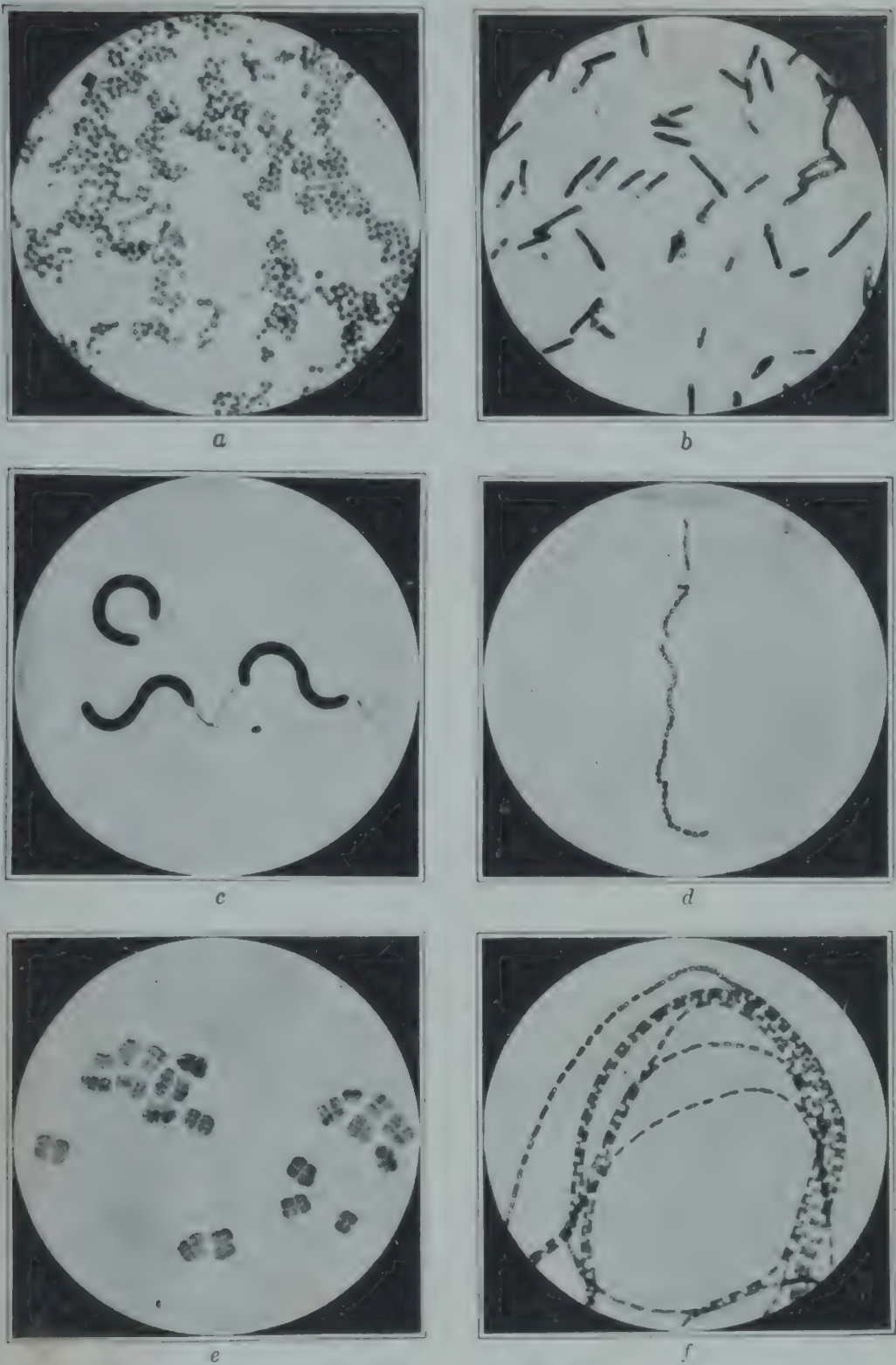


Fig. 50. Photomicrographs of various morphological types of bacteria (*Eubacteri-*  
a. *Staphylococcus*; b. *Clostridium botulinum* showing spores; c. *Spirillum undula*;  
streptococcus; e. *Sarcina agilis*; f. *Bacillus anthracis*. (Photographs by Zettnow.  
printed from Hilliard: *A Textbook of Bacteriology and Its Applications*, Ginn & Co.)

## THE ACTINOMYCETALES

The branching bacteria which appear to be most closely related to the molds belong to the order *Actinomycetales*. This order is subdivided into groups (named Families in the formal classification) according to whether the bacteria are long branching filaments or short rods with only a slight tendency to branch. The **actinomycetes** are examples of the branching filamentous or mycelial type, whereas the **mycobacteria** are nonfilamentous rods which branch only occasionally. The most mold-like of all are the common soil actinomycetes such as the *Streptomyces*, which produce an aerial mycelium bearing conidia. The filamentous actinomycetes are considered to be bacteria and not molds because their filaments are only about  $1\ \mu$  wide, being no larger than some true bacteria, and because there are no nuclei and no cross walls visible in the filaments. All members of the Actinomycetales are nonmotile, gram-positive in young cultures, and most of them are aerobic. Throughout the group there is a tendency toward granular staining. The property of acid-fastness or retention of dyes in the presence of acids characterizes all the mycobacteria and some of the filamentous forms.

**Actinomycetes.** The filamentous actinomycetes are primarily free-living soil bacteria, but they and other closely related filamentous organisms also occur as normal inhabitants of the mouth and throat. A few are pathogenic and chief among these is *Actinomyces bovis*, an anaerobe which causes actinomycosis in man and cattle. In the tissues this organism grows in rosette-shaped colonies with its filaments radiating out from the center in a sunburst design and terminating in thickened "clubs." The name *Actinomyces* appropriately means "ray fungus." Yellowish nodules known as "sulfur granules" are visible to the unaided eye in the pus from actinomycotic lesions, and each granule is composed of one to several of these rosettes of clubbed filaments. Filaments of the pathogenic *Actinomyces* are apt to fragment into short rods (similar to the arthrospores of molds), and, unlike many of the saprophytic types, they do not produce conidia. When stained by Ziehl-Neelsen method (see page 127) *Actinomyces bovis* is readily decolorized in acid alcohol and is, therefore, said to be non-acidfast.

**Nocardia.** The genus name *Nocardia* has persisted in medical literature to designate certain pathogenic, aerobic, filamentous members of the Actinomycetales, and to separate them from the morphologically similar, anaerobic *Actinomyces bovis*. *Nocardia asteroides* is an acid-fast, aerobic, mold-like bacterium isolated from clinical cases indistinguishable from those caused by *A. bovis*. Another aerobe, *Nocardia madurae*, is one of the causes of a tropical disease known as **mycetoma** or **madura foot**. Actinomycosis and nocardiosis are usually considered with the mycoses or diseases caused by the yeasts and molds and not with the bacterial diseases because the clinical and tissue reactions are similar in these infections.

**Streptomyces.** The common saprophytic actinomycetes of the soil are the most mold-like bacteria. Frequently a high percentage of the colonies in a culture made from soil are leathery and difficult to dislodge from the agar, their surface



is often covered with a white or pigmented, powdery film, the surrounding agar may or may not be colored and they emit a distinct odor reminiscent of newly plowed soil. Microscopic examination explains the gross appearance of such a colony; branching filaments form a vegetative mycelium embedded in the agar, and the tips of aerial filaments bear chains of spherical bodies, the conidia, which give the colony its powdery surface. These are the *Streptomyces* which differ from the *Actinomyces* in that they are aerobic, conidia-bearing saprophytes with a non-fragmenting mycelium. It is one of these bacteria, *Streptomyces griseus*, that produces the antibiotic substance streptomycin.

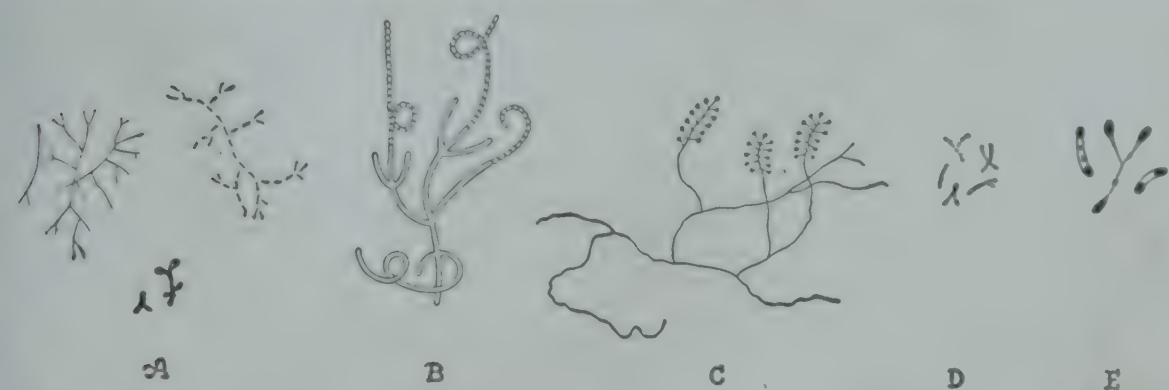


Fig. 51. Morphologic types of branching bacteria, the actinomycetes. A, *Actinomyces*, showing fragmentation of branching filaments; B, *Streptomyces*, showing characteristic chains of conidia; C, *Micromonospora* with conidia borne singly on branching, aerial filaments; D, *Mycobacterium*, short, acid-fast rods which branch occasionally; E, *Corynebacterium*, branching of bacilli in old cultures. (Redrawn from Skinner, Emmons and Tsuchiya: *Henrici's Molds, Yeasts and Actinomycetes*, John Wiley & Sons, and other sources.)

**Mycobacteria.** The aerobic acid-fast bacilli which branch occasionally and frequently exhibit granular staining belong to the genus *Mycobacterium*. The most distinctive property of the mycobacteria is their ability to retain certain dyes such as carbol fuchsin after treatment with acid or acid alcohol. It is difficult for stains to penetrate these cells because of their high lipid content. Only after prolonged treatment with the red dye carbol fuchsin, which is one of the most penetrating stains, or by the application of heat during this staining process, can these bacteria be colored. Once stained, however, the waxy material of the cells holds the carbol fuchsin even in the presence of the acid alcohol. The Ziehl-Neelsen staining technique which tests for acid-fastness is described under microscopic methods (page 127). Bacteria that retain the red color of carbol fuchsin after treatment with acid alcohol are considered to be acid-fast. Few other bacteria can withstand the decolorizing effect of strong acids and those that are decolorized in their presence are said to be non-acidfast. Consequently, acid-fastness offers a valuable clue in the identification of the mycobacteria.

The mycobacteria are slender, usually unbranched, straight to slightly curved rods about 2 to 5  $\mu$  in length. Under the microscope the cells may occur singly, strewn over the field like jack-straws, or they may lie "spoon fashion" in compact

clumps. Their cream to yellow-orange colonies are remarkable for their waxy consistency, the growth of saprophytic strains usually being butyrous and deeply pigmented, while pathogenic varieties commonly produce light buff or cream-colored colonies with a heaped-up, dry, crumbly surface.

Many members of this group, like *Myco. phlei*, the "timothy grass bacillus," and *Myco. butyricum*, the "butter bacillus," live saprophytically in nature, but some are parasitic on a wide range of hosts. *Mycobacterium smegmatis* is a non-pathogenic human parasite of the external genital region. The most important acid-fast bacillus from a medical viewpoint is *Myco. tuberculosis*, different varieties of which cause tuberculosis in man (*Myco. tuberculosis* var. *hominis*), cattle (*Myco. tuberculosis* var. *bovis*) and birds (*Myco. tuberculosis* var. *avium*). There are few animals that are not subject to infection with some species of *Mycobacterium*, even cold-blooded animals like frogs, snakes and turtles being susceptible to diseases resembling tuberculosis. *Mycobacterium leprae*, an acid-fast rod indistinguishable microscopically from the tuberculosis bacillus, is always associated with cases of leprosy and there is strong evidence that it is the cause of this disease.

**Corynebacteria.** The corynebacteria are straight to slightly curved rod-shaped bacteria which are frequently clubbed at one or both ends and which may branch in old cultures. They are not acid-fast, but the cells are characterized by marked irregularity in shape and staining, and for this reason they are said to be highly **pleomorphic**. In *Corynebacterium diphtheriae*, the cause of diphtheria in man, pleomorphism is a regular finding, with the cells ranging from short, almost coccoid, shapes to long slender rods clubbed at one or both ends or swollen in the middle. The irregularity of cell morphology is further emphasized by their reaction to certain stains, particularly methylene blue. Volutin, a reserve food material, is commonly stored in the older cells in large and small **metachromatic** granules, so-called because they stain deeply with methylene blue like the chromatin of higher plant and animal cells, while the rest of the bacterial cell has little affinity for the dye. The large metachromatic granules occupy the swollen or club-shaped regions of the cells. Sometimes this deep staining material occurs in bands rather than granules and then the cells appear barred. Postfission snapping movements cause the cells to form characteristic parallel or palisade arrangements and angular "Chinese letter" groups. The corynebacteria are gram-positive, nonmotile and usually aerobic.

Recently the idea that the corynebacteria should be classified with the true bacteria has gained favor, the reason being that branching is confined to senile cells, and in this respect the corynebacteria are no different from many of the Eubacteriales. There is nothing about the appearance of the gray, cream or yellow colonies of this group to distinguish them from those of ordinary bacteria.

The great majority of corynebacteria that have been studied carefully have been isolated from lower animals and man, but they are also common air contaminants. Certain species parasitize the nose, throat and conjunctivae of normal



Individuals and others are common pathogens in the lower animals. *Corynebacterium diphtheriae* is the one important human pathogen in this group.

### THE CHLAMYDOBACTERIALES

The sheath-forming, alga-like bacteria of the order Chlamydobacteriales are aquatic, filamentous organisms which show no true branching. These bacteria are found in natural waters, particularly those of high mineral content such as that of certain springs and bogs. Included in this order are the sheathed iron bacteria.

**The Sheathed Iron Bacteria.** These bacteria may be filamentous or they may be ordinary size bacterial cells like bacilli or cocci arranged in a long chain. Surrounding the filament or chain of smaller cells, as the case may be, is a tubular sheath about as wide as the largest true bacterium and often several hundred microns in length. The bacteria secrete the mucilaginous material which fashions the sheath and, although its chemical nature differs among the species, the majority produce a sheath of organic matter which soon becomes impreg-

nated with ferric hydroxide. Small, sometimes flagellated cells slip out from the open tip of the sheath to start new filaments. False branching occurs in some species, where short bacillary or coccoid cells push out from the sides of the sheath to start a new filament or chain of bacteria. Some young filaments may be motile, but usually the iron bacteria are sessile, attached to rocks and other objects by part of their sheath, or they are motionless free-floating strands.

None of the filamentous iron bacteria are parasitic. They grow in natural waters, lakes, springs and brooks where decaying vegetation provides a rich supply of iron-containing organic compounds. The iron from such compounds is deposited in the sheaths of the bacteria as ferric hydroxide. Certain of the iron bacteria are **autotrophic**, *i.e.*, they can use inorganic substances for food instead of the usual organic compounds. Such an autotrophic species growing in a medium containing ferrous carbonate produces the following oxidative reaction from which it derives energy:



It is not known whether all iron bacteria gain energy from the oxidation of iron or whether oxidation of ferrous compounds in the sheath may have no relation to

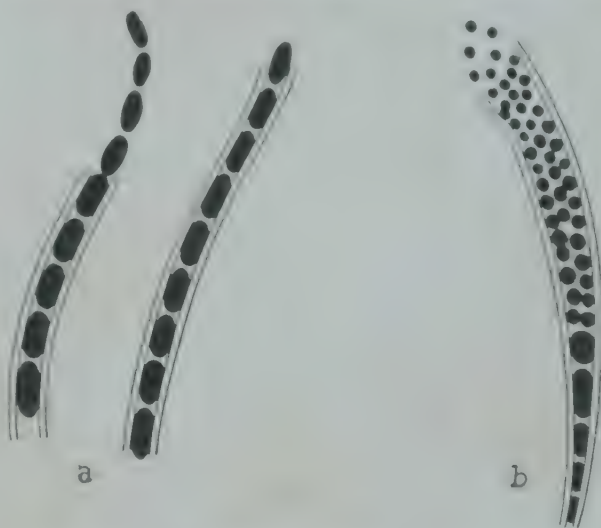


Fig. 52. Examples of sheathed iron bacteria. a. *Leptothrix ochracea*; b. *Crenothrix polyspora*. (Redrawn from Henrici: *Biology of the Bacteria*, D. C. Heath & Co.)

cell metabolism. With age the sheaths become heavily encrusted with ferric hydroxide, the reproductive processes of the bacteria stop and finally they die. As a result, where these organisms grow there accumulates a yellow or red-brown slime, like greasy iron rust, which coats rocks and plants and forms a flocculent deposit on the water bed. It is not difficult to imagine the problem the iron bacteria create once they start growing in metropolitan or private water supplies. Attractive potable water becomes offensive in taste, odor and appearance, and pipes are eventually clogged. The efficiency of sewage disposal systems can also be impaired by the growth of these bacteria. Accumulations of ferric hydroxide formed by the iron bacteria have undoubtedly contributed at least in a small measure to the earth's iron ore deposits.

### THE SULFUR BACTERIA

A variety of morphologically dissimilar bacteria metabolize sulfur and inorganic sulfur compounds. These sulfur bacteria may be subdivided according to (1) whether sulfur granules are stored within the cells and (2) whether they possess photosynthetic pigments. The nonpigmented bacteria which utilize inorganic sulfur but do not store free sulfur in their cells are brought together

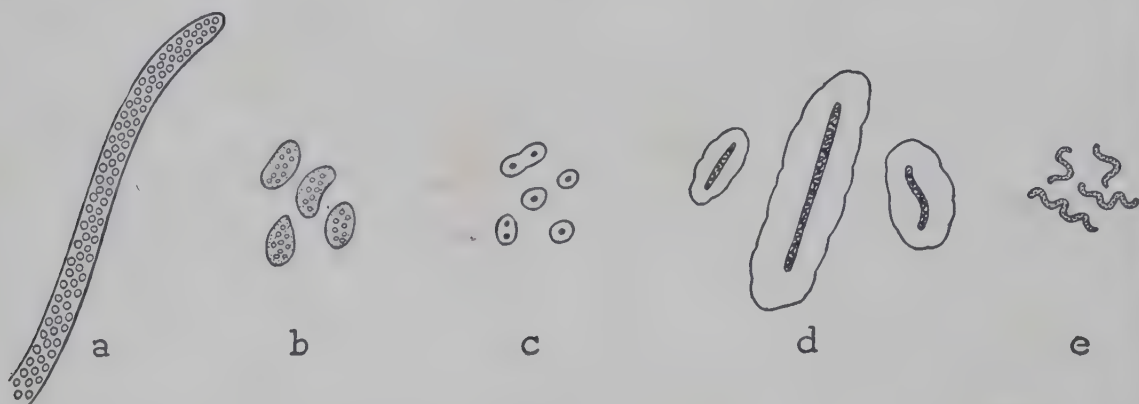


Fig. 53. Examples of sulfur bacteria. a. *Beggiatoa alba*, a filamentous, non-pigmented form which stores sulfur (note granules); b, *Thiophysa volutans*, a sulfur-storing, non-filamentous, colorless form; c, *Rhodotheca pendens* and d, *Rhodocapsa suspensa*, pigmented, non-filamentous sulfur bacteria which do not store sulfur and which form an enveloping slime mass; e, *Rhodospirillum rubrum*, a pigmented sulfur bacterium. (a, b, c, and d redrawn from Ellis: *Sulfur Bacteria*, Longmans, Green & Co.)

in the genus *Thiobacillus* (thio- from *theion*, Greek for sulfur), and they are considered to be true bacteria (Eubacteriales). On the other hand, nonpigmented bacteria that store sulfur granules and the pigmented sulfur bacteria (which may or may not store free sulfur) have been combined in a separate order, the Thiobacteriales. The white sulfur-storing bacteria may be round to oval cells, whose size is greater than that of ordinary bacteria, or large unbranched filaments. The common filamentous sulfur bacteria (*Beggiatoa*) are long nonseptate strands of protoplasm, but in others the filaments



re chains of rod-shaped cells enclosed in a sheath. The group of pigmented sulfur bacteria (*Rhodobacteria*) includes organisms which are morphologically similar to cocci, bacilli, and spirilla of the Eubacteriales except that they contain photosynthetic pigments.

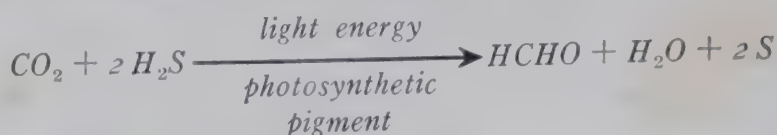
Obviously these bacteria are somehow related in their physiology, *i.e.*, they use inorganic sulfur in their metabolism. According to morphology, however, they could be separated and classified with other orders of bacteria. It has been proposed recently that the nonfilamentous forms be included in the order Eubacteriales and the filamentous sulfur bacteria be placed in the order Thlamyodobacteriales.

**The Filamentous Sulfur Bacteria.** These unpigmented or white, filamentous, sulfur-storing bacteria grow in waters containing hydrogen sulfide and a good supply of air, as in streams from sulfur springs, in bogs and sink drains. Under the microscope a bit of their grayish slimy growth appears as a mass of long, thick, opaque filaments packed with granules. The common forms, such as *Leptothrix alba*, move with a slow gliding motion and their free ends wave gently back and forth. They have been described as looking like bleached-out strands of *Oscillatoria*, a filamentous blue-green alga, and it has been suggested that they are close relatives of these algae, linking the simplest of the green plants with the true bacteria. These white sulfur bacteria are autotrophic; they do not need organic substances for food, but obtain the materials for synthesis of their protoplasm from inorganic compounds and their energy from the oxidation of hydrogen sulfide and sulfur. Since they require atmospheric oxygen to effect the oxidations that set free energy, they are obligate aerobes. Hydrogen sulfide is first oxidized to water and free sulfur with concomitant release of energy. It is this sulfur which accumulates in globules or granules in the protoplasm and which is later oxidized itself with further release of energy. These chemical reactions may be expressed by the following equations:

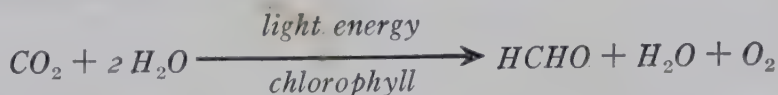


**The Pigmented Sulfur Bacteria.** Many common bacteria elaborate and secrete pigments which tint their colonies and also the surrounding medium if the colored substance is water soluble. In most cases these pigments are incidental and apparently useless by-products of cellular metabolism. There are, however, certain bacteria that possess intracellular pigments which play a major role in their metabolism. These are the chlorophyll-containing, green sulfur bacteria and purple sulfur bacteria. In addition to a bacteriochlorophyll, the purple sulfur bacteria contain a variety of reddish (carotenoid) pigments. The photosynthetic pigments of these organisms capture energy from sunlight and make it available to the organisms they serve. Bacteriochlorophyll is not identical with the chlorophyll of green plants, but it is very similar in function. These pigmented bacteria when grown anaerobically in the presence of sunlight utilize

hydrogen sulfide and carbon dioxide to produce formaldehyde (HCHO). The reaction may be represented by the following equation:



The resulting sulfur may or may not be stored inside the cells, depending upon the species. Hydrogen sulfide, it should be noted, is not the source of the energy here as it is for *Beggiatoa* and other unpigmented sulfur bacteria. As in the photosynthesis of green plants, light is the source of energy for the production of formaldehyde, which is believed to be the first step in the construction of protoplasm from inorganic substances. But whereas carbon dioxide is reduced by the hydrogen of water in the photosynthesis of green plants, in the green and purple sulfur bacteria carbon dioxide is reduced by the hydrogen of hydrogen sulfide. This is evident from a comparison of the following equation, representing the initial step in the photosynthetic process in green plants, with that of bacterial photosynthesis above.



Morphologically the pigmented sulfur bacteria resemble a variety of true bacteria, cocci, vibrios, spirilla and bacilli, and in a newly proposed classification they are included in the Eubacteriales. Like the white sulfur bacteria, the pigmented forms are found in hydrogen sulfide-containing waters, in springs, bogs, tide-pools, swamps, sewage and other liquids where the decomposition of organic matter is accompanied by the liberation of hydrogen sulfide.

### THE STALKED BACTERIA

To those interested in medical microbiology the stalked bacteria (Caulobacteria) are merely a curiosity. They are uncommon free-living organisms found chiefly in water and occasionally in soil. Their cells are like those of the true bacteria but each "spins" a stalk which attaches the bacterium at its tip to a firm object in the water. The stalk material is secreted by the cell and may be of an organic substance, such as a gum, or, as in the case of *Gallinella ferruginea*, it may be composed of ferric hydroxide, the same material found in the sheaths of filamentous iron bacteria. These stalked bacteria are neither filamentous nor ensheathed and the cells do not branch, although it is common for their stalks to do so. A separate order, *Caulobacteriales*, was created for the stalked bacteria, but recently the trend is to consider them with the Eubacteriales.

### THE MYXOBACTERIALES

Another interesting group of higher bacteria is the order *Myxobacteriales* or the slime bacteria. These are free-living bacteria that are found in the forest on



composing organic matter, the droppings of wild animals or the humus of the soil. When such material is incubated under suitable conditions, small moist, yellow or orange-pink colonies develop which are found to be composed of long, slender, flexible, pigmented bacilli with tapering ends, embedded in a slimy matrix. The organisms are motile, not by means of flagella or any other locomotor organelles, but by a creeping or gliding motion similar to that of certain blue-

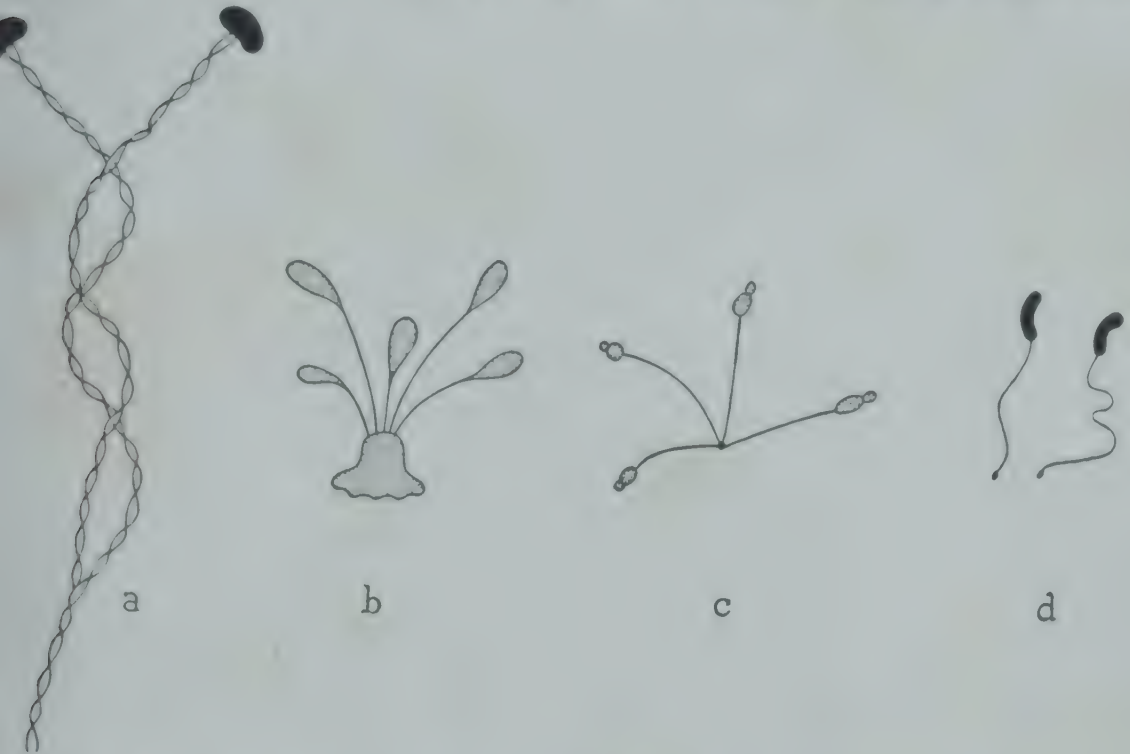


Fig. 54. Examples of stalked bacteria. a, *Gallionella ferruginea*, a bean-shaped bacterium which forms flat, twisted stalks of ferric hydroxide; b, a freshwater bacterium with stalks arising from a mass of amorphous debris; c, budding freshwater bacterium supported by stalks attached to a common holdfast; d, stalked vibrio from lake water. (redrawn from Henrici: *Biology of the Bacteria*, D. C. Heath & Co.; b, c and d, redrawn from Henrici and Johnson, *J. Bact.* 30:91, 1935.)

green algae. All the cells move through the slime at once and in the same direction, and this **swarming** is so characteristic of the vegetative phase in the life cycle that it is termed the **swarm stage**. In this stage the bacteria are actively digesting and utilizing dead organic matter and are reproducing by binary fission.

After several days the vegetative processes slow down and the bacteria swarm outward, forming little knobs at various points along the margin of the colony in preparation for encystment or the beginning of the **resting stage**. The uppermost bacilli shorten into nonmotile, round or oval bodies which are crowded by more and more cells pushing up into the tip of each bulging projection until it is completely packed with rounded reproductive cells. As the slime dries, the mature cyst becomes detached and can then be disseminated by wind or water. The developing cysts of some species are supported by slime stalks which may

become elaborately twisted or branched. Later under proper conditions of growth the cysts become soft and the enclosed cells form a new slime colony of swarming bacilli which repeat the cycle. The type of locomotion in these flexible cells, their slimy vegetative growth and peculiar life cycle set the Myxobacteria apart from the true bacteria.

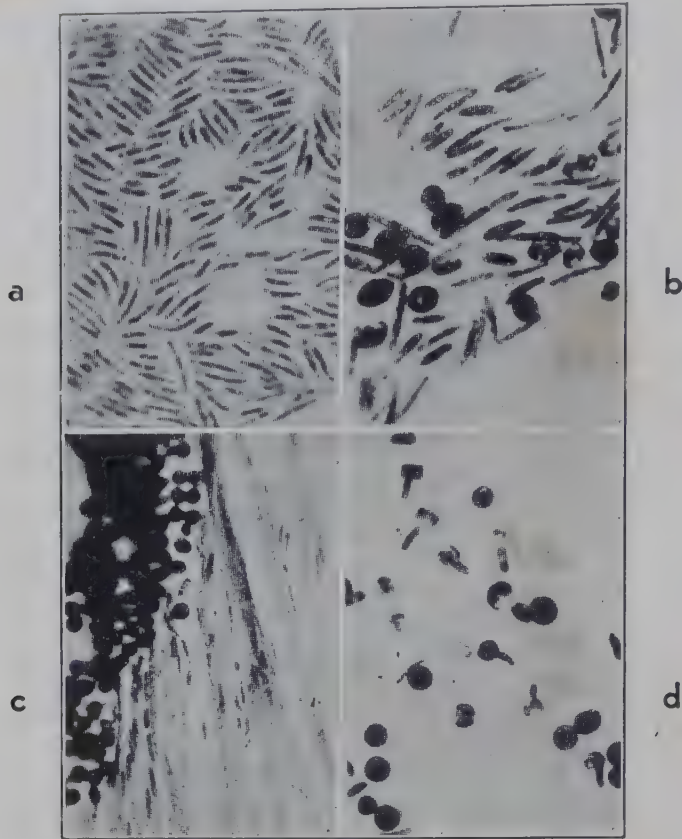


Fig. 55. Stages in the life cycle of a myxobacterium, *Myxococcus xanthus*. a, Vegetative cells of the swarm stage; b, beginning of the resting stage, spore-formation; c, spores on the surface of the slime mass in which a few vegetative cells may be seen; d, germinating spores giving rise to vegetative cells of the swarm stage. (Magnification approximately  $\times 1,000$ .) (From Beebe: *J. Bact.* 42:193, 1941.)

### THE SPIROCHAETALES

Spirochetes are long, slender, flexible, tightly coiled to loosely spiral organisms that in some respects resemble protozoa and in others are like the true bacteria. The majority are aquatic saprophytes which live in fresh water, seawater or in sewage, although some are parasites of animals and man. A few may cause important human disease. The parasitic spirochetes have been confused with some of the blood-dwelling protozoa which are similarly flexible motile organisms and also with the spiral true bacteria of the genus *Spirillum*. A brief description of the spirochetes and a comparison of them with the true bacteria and with the protozoa will show the reasons why they are at present classified as protozoan-like, higher bacteria.



The name "spirochete," meaning coiled hair, nicely suggests the appearance of these organisms whose width is always very slight (0.25 to 1.5  $\mu$ ) in relation to their length (6 to 200  $\mu$  or longer). However, they are never filamentous, for the living cells are perpetually twisted into the typical wavy or spiral forms. All spirochetes are motile either by a serpentine creeping movement or by a swift rotary movement due to contractions of the cell that drive it ahead like an animated corkscrew. Reverse rotation along its spirals propels the organism in the opposite direction. The flexibility and contractility of the spirochetes are the chief distinguishing features which separate them from the true bacteria.

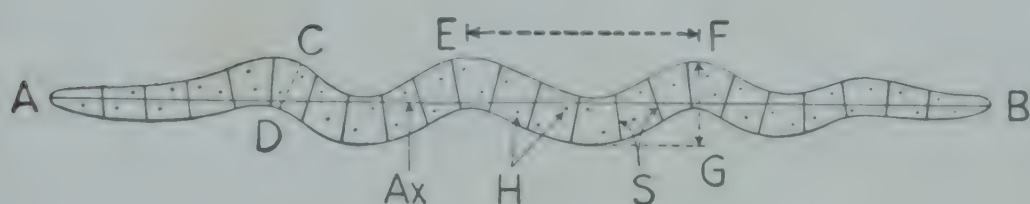


Fig. 56. Diagram of a typical spirochete. AB, Length; CD, width; EF, spiral length; FG, spiral depth; Ax, axial filaments; S, septa; H, metachromatic granules. (From Belding and Marston: *A Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)

Locomotion of the curved true bacteria is supposed to be effected by the action of flagella and not by movement of the cells themselves. Although in some spirochetes the protoplasm at the ends of the cells extends into fine terminal filaments that resemble flagella, it is believed that movement is due to contractions of their cell bodies and not to these terminal filaments. However, what appear to be genuine flagella have been demonstrated on certain spirochetes in photographs made by the electron microscope, and other recent investigations of bacterial flagella have reopened the whole subject of the mechanism of bacterial motility. It may be that present ideas will have to be revised. (See page 92.)

Many spirochetes do not stain readily with bacteriological stains but are colored more successfully by those which stain the protozoa. Some, including the spirochetes of the mouth, take the ordinary aniline dyes such as methylene blue and gentian violet. Their reaction to the Gram stain is always negative. Special silver impregnation methods are commonly used to demonstrate spirochetes in sections of infected tissues such as those taken for postmortem examination. When possible it is preferable to avoid staining techniques and to examine the living spirochetes against a dark background as in the darkfield illumination of fresh wet preparations. Thus exudate from a suspected primary lesion (chancre)

in syphilis is examined under a darkfield microscope to determine the presence of the causative spirochete (*Treponema pallidum*) and to establish the diagnosis.

Our knowledge of the spirochetes has been definitely hampered by difficulties which accompany attempts to cultivate them. As a matter of fact, only a few parasitic spirochetes have been grown outside the animal body. Of those that have been cultivated, some are aerobes and others are obligate anaerobes.

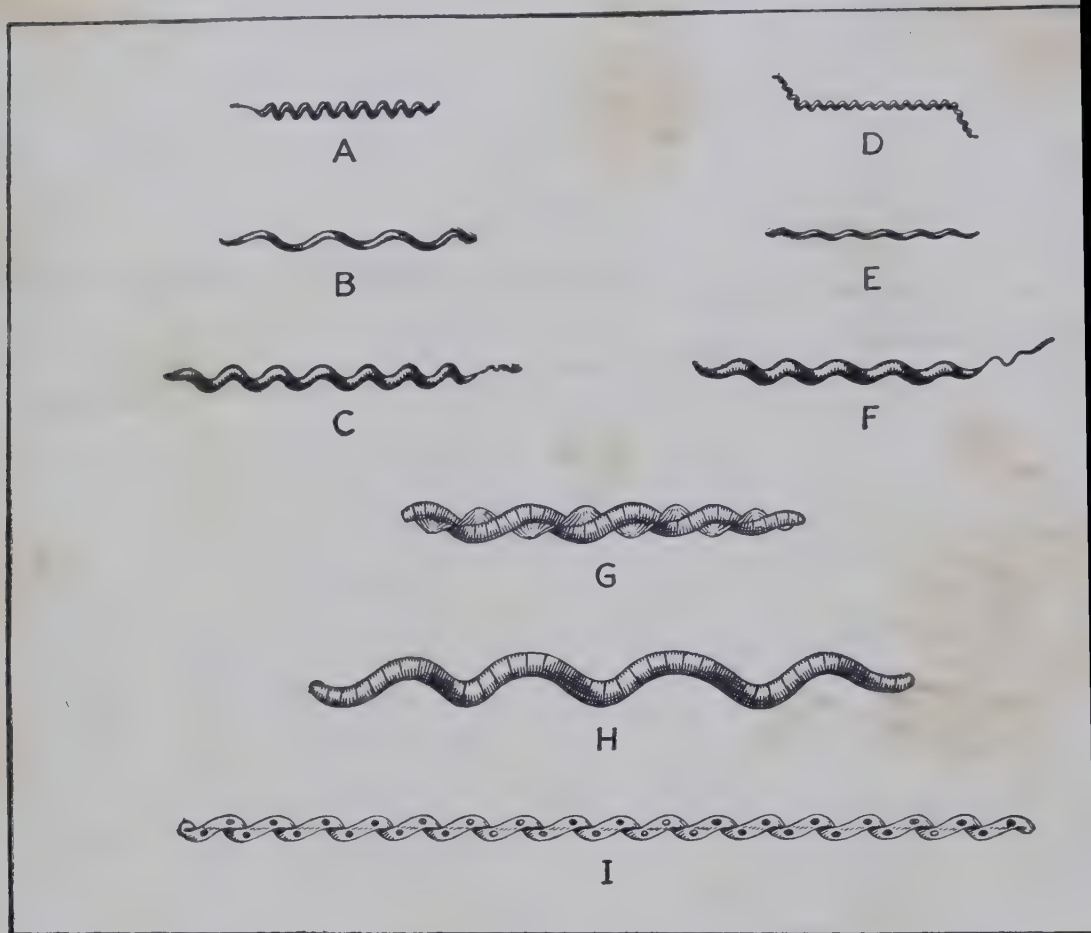


Fig. 57. Varieties of spirochetes. A, *Treponema pallidum* ( $\times 2,000$ ); B, *T. vincentii* ( $\times 2,000$ ); C, *Borrelia refringens* ( $\times 2,000$ ); D, *Leptospira icterohaemorrhagiae* ( $\times 2,000$ ); E, *Treponema (Borrelia) macrodentium* ( $\times 2,000$ ); F, *T. recurrentis* ( $\times 2,000$ ); G, *Cristispira* ( $\times 500$ ); H, *Saprospira* ( $\times 500$ ); I, *Spirillum* ( $\times 500$ ). (From Belding and Marston: *A Textbook of Medical Bacteriology*, Appleton-Century Co., Inc.)

There is wide variation in size among the spirochetes. In general, the free-living species are large for bacteria, approaching or exceeding  $100 \mu$  in length, whereas the parasitic spirochetes are about 6 to  $14 \mu$  long and less than  $1 \mu$  wide. Some of the finest, highly motile forms (*Leptospira*) can make their way through filters which hold back most bacteria.

Features of the spirochetes which differentiate them from true bacteria strongly suggest kinship to the protozoa. But protozoa have one or more distinct nuclei and no distinct nucleus has been demonstrated in the spirochetes.



Furthermore, trypanosomes, the protozoa which bear closest resemblance to the spirochetes, reproduce by longitudinal binary fission, whereas spirochetes divide by transverse fission as do the rod-shaped bacteria. This is important evidence which, considered together with their size and shape, relates the spirochetes to the bacteria.

Six different genera of spirochetes have been described which differ from one another in such traits as habitat, size, cell structure and pathogenicity. A brief outline of these genera with some of their more important characteristics is presented in Table 3. Other features helpful in the identification of a spirochete besides those mentioned in the table are shape of the cell ends, the width and length of each spiral, the number of spirals and the character of its movements.

TABLE 3. MORPHOLOGY AND PATHOGENICITY OF SPIROCHETES

GENUS NAME	HABITAT	APPROXIMATE SIZE	CELL STRUCTURE	DISEASES PRODUCED BY CERTAIN SPECIES
<i>Spirochaeta</i>	Fresh or sea water; sewage	$0.5 \times 150 \mu$	Protoplast coiled around axial filament; no terminal filaments	Nonpathogenic
<i>Cristispira</i>	Commensals in oysters, clams, fresh-water mussels	$1.0 \times 70 \mu$	Ridge-like membrane or crista wound around cell body; no axial or terminal filaments	Nonpathogenic
<i>Saprospira</i>	Free-living in water	$1.0 \times 80 \mu$	Like <i>Cristispira</i> except no crista	Nonpathogenic
<i>Leptospira</i>	Parasites of rats, dogs, and man; may be free-living in water	$0.25 \times 7 \mu$	No axial or terminal filaments; cells hooked at one or both ends	Infectious jaundice (Weil's disease)
<i>Treponema</i>	Parasites of man and animals	$0.25 \times 10 \mu$	Axial filament runs through center of protoplast; terminal filaments present	Syphilis Yaws
<i>Borrelia</i>	Parasites of man and animals	$0.35 \times 16 \mu$	Differ from <i>Treponema</i> in size and character of spirals	Relapsing fever Associated with other bacteria in Vincent's angina and similar infections

The medical microbiologist's interest in spirochetes is confined usually to the *Leptospira*, *Treponema* and *Borrelia* (see Table 3). The nature of the important

pathogenic species will be more fully described in later discussions of the diseases they produce. The *Treponema* and *Borrelia* are so similar that the advisability of separating them into two different genera has been questioned, and since there is not complete agreement here the names *Treponema* and *Borrelia* are often used by different authors to indicate the same organism. Practically every normal human mouth harbors the spirochetes *Treponema macrodentium*, *T. microdentium*, *T. mucosum* and *Borrelia vincentii*, while another, *T. refringens*, appears to be a harmless parasite of the genitalia.

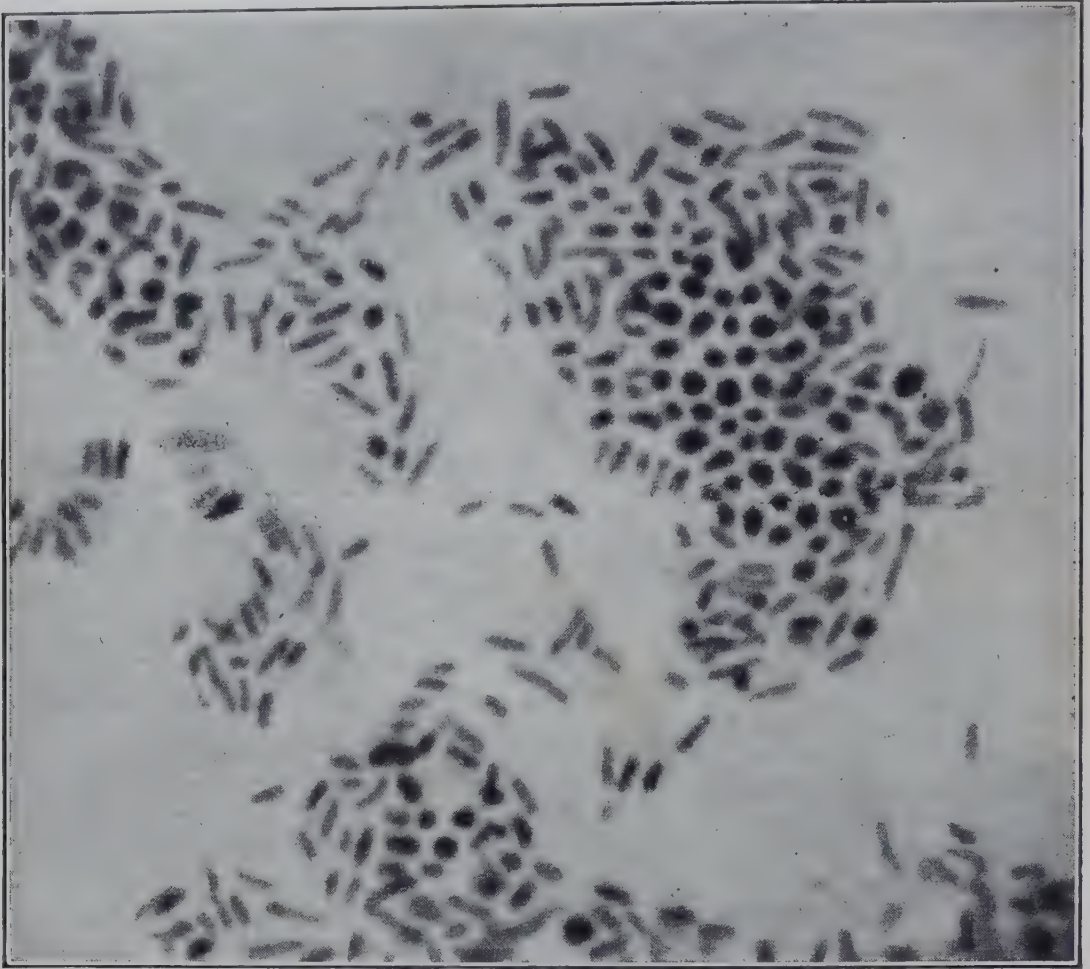


Fig. 58. A pleomorphic bacillus, *Pasteurella tularensis*, showing coccoid and bacillary forms in the same field. (Magnification approximately  $\times 5,000$ .) (From the Army Institute of Pathology, Neg. No. 37206, Courtesy of Dr. Edward Francis, U.S.P.H.S.)

#### PLEOMORPHISM AND INVOLUTION FORMS

The cell morphology of all bacteria varies with the age of the culture and the nature of the culture medium as well as other environmental factors. Bizarre, scarcely recognizable forms are likely to develop in old cultures or under unfavorable growth conditions which hasten the death of the culture. These abnormal cells are known as **involution forms**. Cocci may become greatly swollen, bacilli may bend or branch, and changes in the internal structure may appear which



cause the cells to look like ghosts of their former selves. While the majority of such cells are probably dead or dying, some may be alive and capable of reproduction under suitable circumstances.

It is characteristic for the cells of certain species to assume a variety of sizes and shapes throughout the development of a culture. This phenomenon of instability of form in a pure actively growing culture is known as pleomorphism, and the bacteria are said to be pleomorphic, *i.e.*, many-formed (Fig. 58). Many of the true bacteria show this trait. Cells of several shapes and sizes may be seen in a single preparation taken from a culture of such an organism, but at different ages of the culture a particular form may predominate. As previously stated, variability of form and occasional branching in old cultures characterize the corynebacteria, including the highly pleomorphic diphtheria bacillus.

# 7

## THE STRUCTURE OF THE BACTERIAL CELL

Our knowledge of the anatomy of the bacterial cell is limited by its minute size and our range of vision. With the invention of ever higher powered micro-

scopes and the introduction of newer microscopic methods some of these limitations have been overcome. A considerable store of information has been accumulated, but there is still much to be learned of the nature and functions of the finer structures of the bacterial cell.



Fig. 59. Electron micrograph of nucleus of *Bacillus mycoides*. Note the appearance of simultaneous division of cell and nucleus. (After Knaysi and Baker, *J. Bact.* 53:539, 1947. From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Appleton-Century-Crofts, Inc.)

From the study of other plant and animal cells we might expect the protoplasm in a bacterium to be organized into a nucleus and cytoplasm, but the organization of the bacterial cell, particularly of its nucleoplasm, is a controversial issue. It has been clearly demonstrated that a bacterium has a cytoplasmic membrane, that it is enclosed in a nonliving cell wall and that a slime layer, which in some instances is thick enough to be seen as a capsule, surrounds this wall. Motile bacteria possess one or more flagella which extend out from the cell wall, but their exact origin and relation to motility are debated. There is recent evidence that they arise in the cytoplasm of the cell and penetrate the cell wall. From a maze of conflicting evidence and incomplete information generalizations are difficult to make. The student should

realize that in the summary of the structure of the bacterial cell here presented, many of the generally accepted ideas are subject to change as investigators contribute new findings. The trend of recent evidence indicates that the bacterial cell is comparable to the cells of other fungi.



**Nucleus.** Whether or not the bacterial cell contains a nucleus has long been a disputed question and the subject of extensive investigation. In any discussion of this matter, one must decide at the outset what is meant by a nucleus. Embedded in the cytoplasm of almost all plant and animal cells lies a dense body of specialized protoplasm with definite structures, including a limiting membrane and the linen threads bearing deep-staining chromatin granules which at the time of mitotic cell division become the chromosomes. This is the physical body recognized as the nucleus of these cells. "Is there a nucleus in the bacterial cell?" If this question refers to a well defined body such as the one just described for the cells of higher organisms, the answer is "No." If, however, by a nucleus one means a mechanism by which hereditary traits are passed from one generation to the next, then the proposition is entirely different, for certainly the bacterial cell has some system which performs the functions usually attributed to a nucleus. Therefore the problem is: What kind of a mechanism have the bacteria for accomplishing these functions?

From time to time and from different points of view it has been claimed that (1) the bacterial cell contains **no nucleus**; (2) that it is **all nucleus** because the entire cell usually stains deeply with dyes which characteristically stain the nucleus in more highly organized cells; (3) that the chromatin material is distributed uniformly throughout the cytoplasm as a **diffuse nucleus**; (4) that there is a nucleus in the bacterial cell, but that it differs from the nuclei of higher organisms in that it can exist in a **diffuse form** or in the form of **nuclear granules**; (5) that the bacterial nucleus is a **single chromosome** which takes the form of a small granule or rod-like body. A number of investigators, using the tools of micro-chemistry, nuclear staining techniques and ultraviolet and electron microscopy, have recently demonstrated in certain bacteria a single granule of nuclear type nucleoprotein which divides before cell division (Fig. 60). This, they believe, represents the bacterial nucleus.

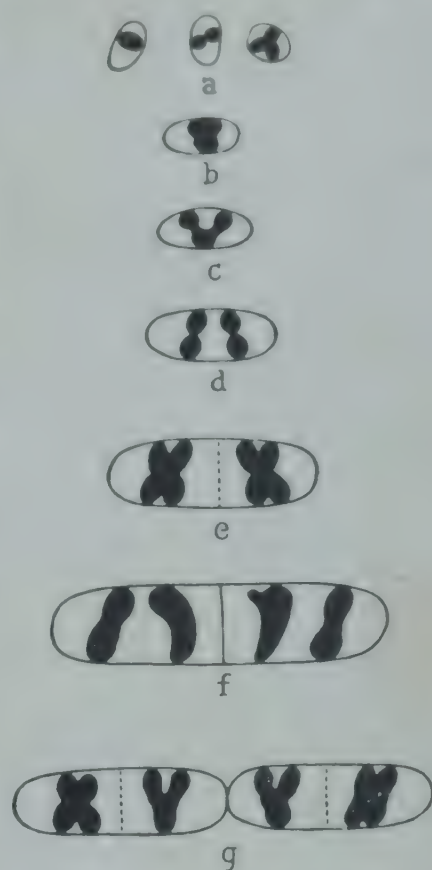


Fig. 60. Diagram of growth and division of chromatinic structures believed to be chromosomes ("nucleoids") in cells of a very young culture of *Escherichia coli*. Cells treated with hydrochloric acid before being stained with Giemsa's solution.

a. Cells found in an 18-hour culture and during the first hour after transfer to fresh agar plate. Note single nuclear structure in these small resting cells.

b-g. Growing cells in young cultures showing resting and dividing nuclear structures.

(Redrawn from Robinow in Dubos: *The Bacterial Cell*, Harvard University Press.)

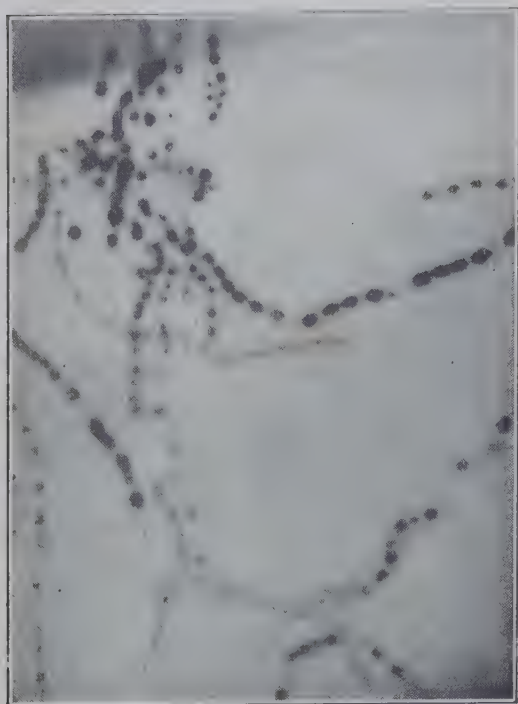


Fig. 61. Free-living spirochete stained with methylene blue to show volutin granules. ( $\times 2,250$ .) (Dyar, M. T., *J. Bact.* 54:483, 1947.)

plays an important role in controlling the kinds of substances that enter and leave the cell, and in determining the amounts of each substance which shall pass through at a given time. Since it is not permeable to all molecules, but exerts a **selective action**, the cytoplasmic membrane is said to be **semipermeable**.

The protoplasm of most bacteria stains evenly, but in some species granules and vacuoles are seen as deep-staining or light areas in the cells. These are **cytoplasmic inclusions** of reserve materials, chiefly excess carbohydrates, fats and volutin, a substance rich in nucleoproteins, which have accumulated and are stored in aging cells. Young actively growing cells usually have no such reserve materials and stain uniformly. Carbohydrates are generally stored in the form of glycogen or a compound closely related to glycogen, but a starch-like substance, iogen, has also been found in some bacteria. When treated with iodine, granules of glycogen stain reddish brown and iogen gives a blue color. The cells of certain bacteria, such as *Spirillum*, may appear vacuolated due to the presence of fat bodies. Droplets of fat are colorless and highly refractile when the cells are treated with regular bacterio-

**Cytoplasm and Cytoplasmic Inclusions.** A homogeneous semifluid substance occupies the space inside the cell wall of the bacterium and this substance is similar to the cytoplasm of the cells of higher organisms. As in other cells, the protoplasm is surrounded by a membrane, the **cytoplasmic membrane**, which separates it from the cell wall. The cytoplasmic membrane is a living structure, for it is actually the living surface of the cell, the region where the protoplasm contacts the environment. Through this membrane materials pass between the cell and the outside world. Digested foods, salts and gases dissolved in water enter the cell, while water, metabolic wastes, exoenzymes and, in some cases, soluble toxins leave the cell by way of this membrane. As in other organisms the exchange of goods between the cell and its surroundings is no haphazard affair. The cytoplasmic membrane

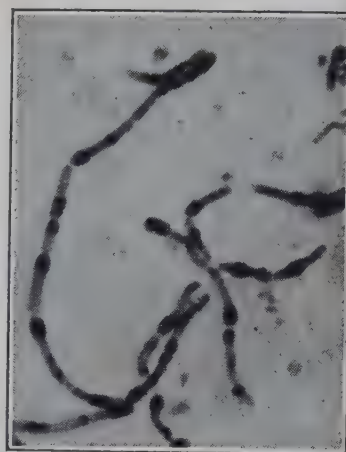


Fig. 62. Photomicrograph of *Bacillus cereus* showing droplets of fat stained with Sudan Black—safranin. (From Burdon, Stokes and Kimbrough: *J. Bact.* 43:717, 1942.)



logical stains, but special fat stains may be used to demonstrate their chemical nature. The volutin or metachromatic granules of bacteria, like those seen in many other fungi, algae and protozoa, stain deeply with methylene blue and other basic aniline dyes. The peculiar property of storing free sulfur distinguishes most of the Thiobacteriales. These sulfur globules dissolve in absolute alcohol, carbon disulfide and certain other solvents, but not in water or hydrochloric acid.

**Cell Wall.** The protoplasm of the bacterial cell appears to be enclosed in a thin, firm, though somewhat elastic, wall of nonliving material. Evidence of such a cell wall has been contributed by studies using the electron microscope, by plasmolysis experiments which cause the living cell substance to shrink away from the wall, by special staining methods and other techniques. The cell wall is not revealed by the usual microscopic preparations, although the fact that most bacteria retain definite shapes is evidence in favor of such a firm, limiting, external structure. Only in the case of some of the higher bacteria, particularly the spirochetes, does the wall allow real flexibility of the cell body. The cells of motile bacilli may bend as they move, but at rest the cell assumes its straight rod shape. The exact chemical composition of the cell wall is at present unknown.

**Capsules.** In many saprophytic and parasitic bacteria another nonliving, outer layer is discernible. This is a capsule of viscous or gummy material that envelops the bacterial cell. Although definite capsules have been demonstrated only in certain bacteria, it is likely that all bacteria have a slime layer which in many cases may be too thin to be detected readily. In addition, a number of species which ordinarily do not produce visible capsules may do so when grown under certain conditions. Furthermore, heavily encapsulated organisms, such as the pneumococcus, may fail to produce a capsule when grown in the absence of body tissues or fluids. Whether the capsular substance is secreted by the bacterium or whether it is the result of some alteration of the cell wall is not known. Usually its chief constituents are various carbohydrates, especially polysaccharides. The chemical nature of the capsular substance appears to be different in different species and even in subgroups or types within a single species.



Fig. 63. Electron micrograph of *Bacillus cereus* showing colorless cell wall enclosing chain of bacilli. (After Johnson, *J. Bact.* 44:551, 1944. From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 6th ed., Appleton-Century-Crofts, Inc.)

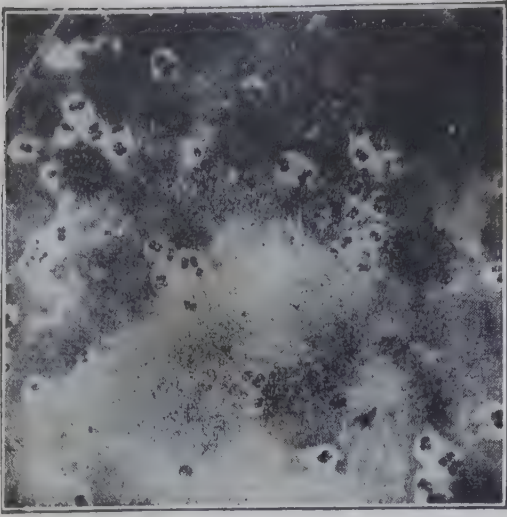


Fig. 64.

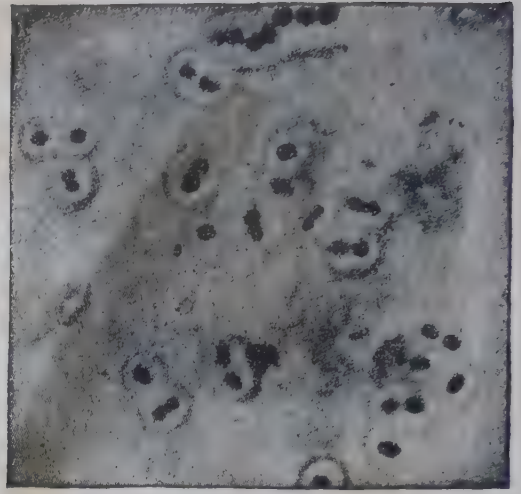


Fig. 65.

Fig. 64. *Micrococcus tetragenus* showing capsules surrounding tetrads of cocci. (From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Appleton-Century-Crofts, Inc.)

Fig. 65. Capsules of pneumococci (*Diplococcus pneumoniae*). (From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Appleton-Century-Crofts, Inc.)

Many nonpathogenic bacteria are encapsulated, but the bacterial capsule is medically significant because encapsulated bacteria are often among the most pathogenic organisms and because resistance to infection may be dependent upon

an immune reaction against the capsular material. Furthermore, certain pathogenic bacteria are classified or typed on the basis of their specific capsular substance. *Diplococcus pneumoniae*, commonly known as the pneumococcus, is the most important species to be divided into types on the basis of the chemical nature of the capsule. At present 74 types of pneumococci have been recognized by serological tests, which reflect the diversity of their polysaccharide capsular components.

A technique for demonstrating bacterial capsules is described in a later section under microscopic methods (see page 128).

**Flagella and Bacterial Motility.** Some of the true bacteria are motile and others are nonmotile. In general,



Fig. 66. An encapsulated streptococcus (*Leuconostoc mesenteroides*).  $\times 1,092$ . (From McCleskey, Faville and Barnett: *J. Bact.* 54:701, 1947.)



cocci are nonmotile, whereas the spirilla are usually motile and the rod-shaped bacteria vary in this respect. Naturally, only living bacteria exhibit true motion, and to study this property, unstained organisms from young actively growing cultures must be observed in a liquid mount, generally a hanging drop (see microscopic methods). The optimal time to examine a culture for motility varies



Fig. 67. Peritrichous flagella of the proteus bacillus (*Proteus vulgaris*). (Kral.)

with the species of bacterium and the growth conditions, but motile bacteria are usually most active in cultures which have been incubated for from 6 to 18 hours. After this time the cells become less active and no motility is seen in old cultures. True motility must be distinguished from the passive movement of microscopic particles caused by evaporation currents and from Brownian movement. All light-weight, microscopic bodies, including bacteria, particles of debris and the like, jiggle or oscillate in place when suspended in a liquid. Only truly motile cells can progress as individuals from one place to another in the droplet. Free-swimming bacteria are believed to move by means of the contractions of

one or more flagella, long slender projections arising from the ends or from various points on the sides of the cells. In the case of the spirilla and a limited number of bacilli, a single terminal flagellum or a tuft of several terminal flagella

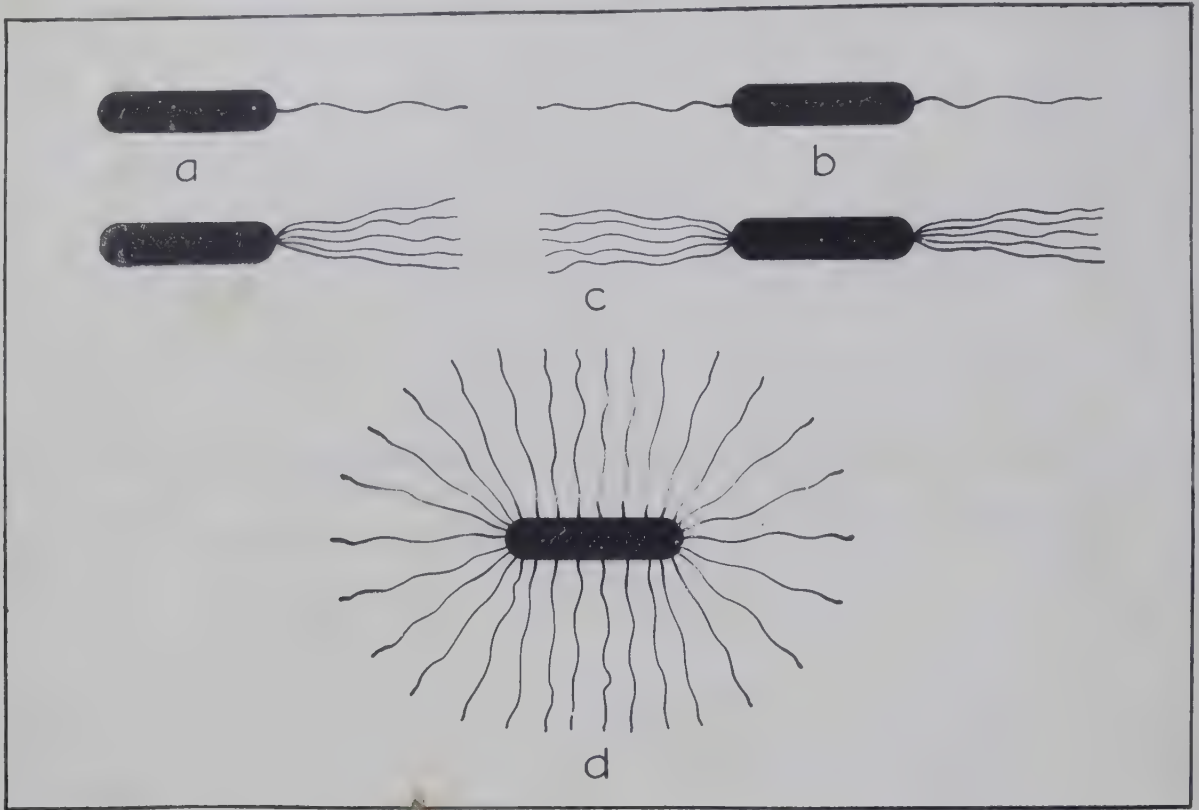


Fig. 68. Arrangement of flagella on bacteria. a, Monotrichate; b, amphitrichate; c, lophotrichate; d, peritrichate. (From Belding and Marston: *A Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)

are located at one or both ends of the cells. Most motile rod-shaped bacteria are equipped with lateral flagella which may number as few as four or five to as many as fifty or more depending on the species of the organism. The following terms are used to indicate the number and arrangement of flagella on the bacterial cell:

**Monotrichate**, a single terminal flagellum

**Lophotrichate**, a tuft of a number of terminal flagella

**Amphitrichate**, one flagellum at each end

**Peritrichate**, several to many lateral flagella

Recently the function of flagella as locomotor organelles has been questioned (Pijper, 1946, 1947), and it is suggested that motility is due "not to the activity of the so-called 'flagella,' but to a gyrating and undulating movement of the bacterial body itself." The flagella are pictured as twisting threads thrown off from the outer slime layer of the bacterium as a result of the rapid spiral movement of the cell body. This idea proposing flagella as artefacts has drawn the fire of other investigators who offer new evidence that flagella are definite entities,



that they originate in specialized structures in the cell body proper, and that their action is responsible for movements of the bacteria (Conn and Elrod, 1947; Kingma Boltjes, 1948).

**Endospores.** Rod-shaped bacteria of two genera, *Bacillus* and *Clostridium*, produce endospores. The vegetative cells of these organisms resemble those of

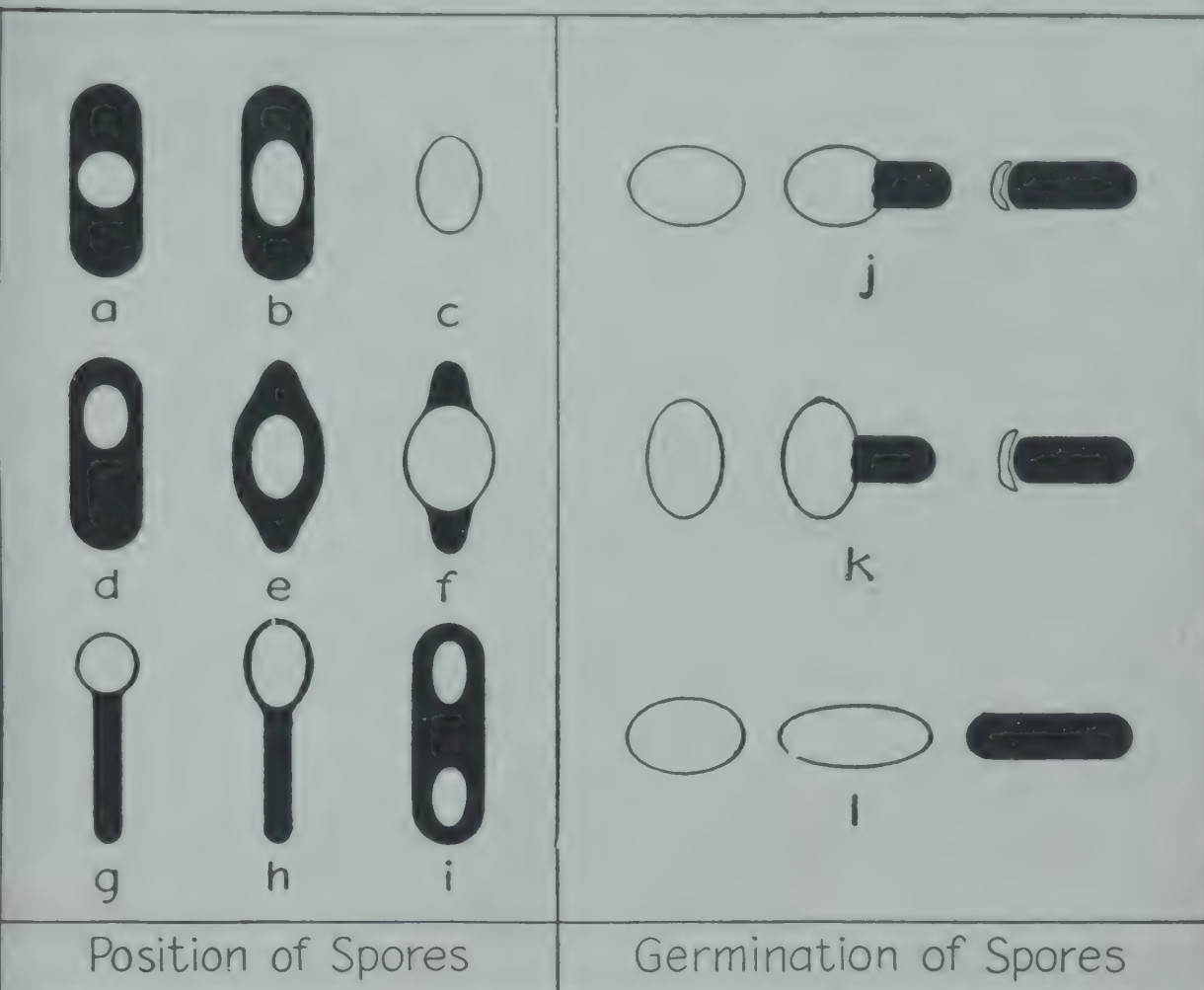


Fig. 69. Position of spores in bacterial cells and types of germination. a. Spherical equatorial; b. oval equatorial; c. spore free from cell; d. oval subterminal; e. oval equatorial with moderate cell-distortion; f. oval equatorial with marked cell-distortion; g. spherical terminal; h. oval terminal; i. two subterminal spores (rare); j. stages of polar germination; k. stages of equatorial germination; l. stages of germination by absorption of spore membrane. (From Belding and Marston: *Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)

the nonsporulating bacteria in the actively growing and reproducing stage, and in young cultures the cells of *Bacillus* and *Clostridium* usually appear as large, even-staining, gram-positive rods. However, as the culture ages smears stained by ordinary methods show cells containing a clear, unstained, round to oval body inside some of the cells. This is the **endospore** which grows until it may occupy the entire width of the cell or even cause the cell to bulge. It may be located in the center (central spore), at the end (terminal spore) or near the end of

the cell (subterminal spore). The old vegetative cell which contains the endospore is considered to be a spore case or sporangium, and the shape of the sporangium, *i.e.*, whether it is swollen or not, as well as the position and form of the endospore, is typical in a given species. When the endospore is mature the sporangium disintegrates and sloughs off, leaving a **free spore**. In old cultures most of the cells are often in the free spore stage.

The endospore is the result of a condensation of essential cell substance with the subsequent development of a highly resistant wall around the condensed

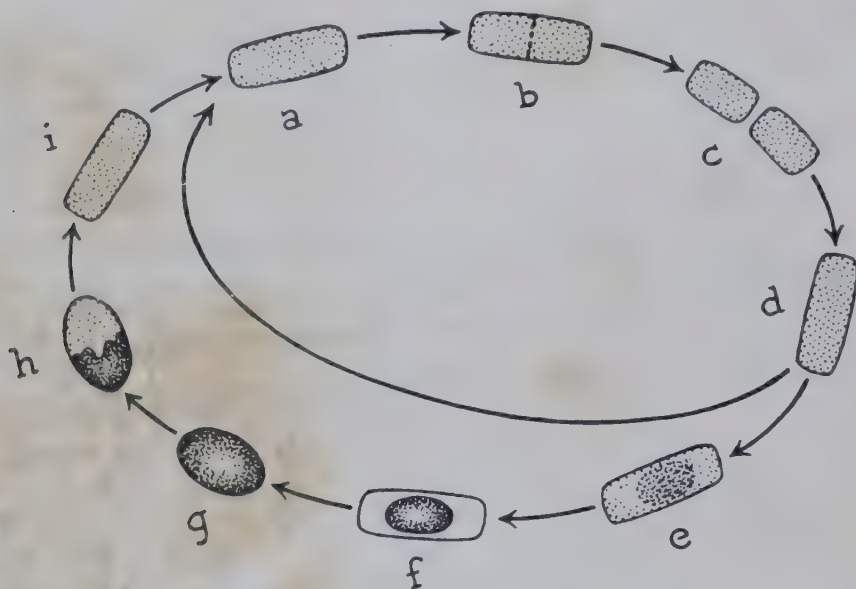


Fig. 70. Life cycle of a spore-forming bacillus. a-d. Vegetative cells reproduce by binary fission for a number of generations; e-i, endospore formation and germination. a, Mature vegetative cell; b, cell division; c, two young daughter cells; d, growing young cell; e, formation of endospore in mature cell; f, mature endospore; g, spore freed from old vegetative cell; h, germination of spore; i, young vegetative cell. (Modified after Hilliard: *Textbook of Bacteriology and Its Applications*, Ginn & Co.)

mass. There have been reports of division of bodies believed to be nuclei in the vegetative cell preceding sporulation followed by incorporation of one of these bodies in the endospore. Except in rare cases only a single endospore is formed in a bacillus and each spore upon germination produces one vegetative cell which grows and multiplies as did the parent vegetative bacillus. The function of the bacterial spore is not then one of reproduction, but rather one of survival and cellular reorganization. Sporulation occurs in a culture at the end of a period of maximum growth and multiplication. It is known to be enhanced by exhaustion of nutrients and influenced by such factors as aeration, temperature and calcium content of the medium.

Bacterial spores are the most resistant forms of life known. Adverse conditions such as drying or exposure to high temperatures and bactericidal chemicals which are sufficient to destroy the vegetative cell have little or no effect on the spore. Bacteria may remain alive in the spore state for months or years in labora-



dry cultures, on objects and in dust and soil. However, the spores of all species of *Bacillus* and *Clostridium* are not equally resistant, as is illustrated by the following data on the length of time various spores survive boiling:

<i>Clostridium welchii</i>	— 5 minutes
<i>Bacillus anthracis</i>	— 10 minutes
<i>Clostridium sporogenes</i>	— 10 minutes to 2½ hours
<i>Clostridium tetani</i>	— 15 minutes to 1½ hours
<i>Clostridium botulinum</i>	— 3 to 4 hours

Certain spores are said to be alive after as long as 16 hours' exposure to boiling temperatures. In general the sporulating bacteria are saprophytic inhabitants of the soil. However, some species, such as those causing anthrax, tetanus and gas gangrene, are highly pathogenic.

A special method of staining bacterial spores is described in the section under microscopic methods.

#### Relation of Cellular Structure and Morphology to Colony Morphology.

The appearance of a bacterial colony is in part determined by the physical and chemical nature of the cells in it. The size and shape of the bacteria, their cell grouping and whether or not they possess capsules and/or flagella are important factors in the formation of the colony, being reflected in its size, shape, elevation, consistency and the character of its edges. They are not, however, the only factors, for physiological traits of the organism such as its growth requirements, rate of reproduction and metabolic products including pigments also contribute to its colony morphology. This subject will be considered again in the discussions of bacterial variation and methods for identifying bacteria (Chapter 12) and bacterial variation (Chapter 17).



Fig. 71. Electron micrograph showing newly germinated spore with portions of disrupted spore capsule remaining on the ends of the bacillus. (After Knaysi, Baker and Hillier: *J. Bact.* 53:525, 1947. From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Appleton-Century-Crofts, Inc.)

## 8

# THE RICKETTSIAE AND VIRUSES

## THE RICKETTSIAE

In 1910 Howard Taylor Ricketts demonstrated that Rocky Mountain spotted fever, a disease originally recognized in the western United States, was transmitted by the bite of the wood tick and that "diplococcoid bodies" occurred in the tissues of the infected ticks as well as in those of infected laboratory animals and man. In later studies he reported the presence of short bacillary forms in the blood of patients suffering from Mexican typhus fever and in the intestinal contents of lice infected with this disease. His observations were soon substantiated by von Prowazek and others, and in 1916 da Rocha-Lima named the agent of typhus fever *Rickettsia prowazeki* in honor of Ricketts and von Prowazek, both of whom had died of typhus fever during their investigations. The generic name *Rickettsia* has since been applied to all similar microorganisms.

**Nature of Rickettsiae.** The rickettsiae (sing., rickettsia) are small bacteria-like organisms which commonly live, often as intracellular parasites, in insects, ticks, mites and other arthropods. In these hosts the rickettsiae multiply chiefly in the cells lining the alimentary tract, but they may occur in the cells of other regions and may also be found free in the intestinal contents. Although certain rickettsiae are pathogenic for their arthropod hosts, many cause them little or no apparent damage, and in some cases the association between the microorganism and the arthropod is so perfectly adjusted that all members of the species are constantly infected.

Certain species of rickettsiae are pathogenic for man and higher animals, producing diseases which, with rare exception, are acquired by the bite of an infected arthropod or by contamination of the bite wound by its feces. Congenital transmission of some pathogenic and nonpathogenic rickettsiae occurs, the microorganisms being transmitted from one generation to the next by way of the infected egg. The rickettsiae are visible by ordinary microscopic methods. With one known exception (*R. burneti*) they do not pass bacterial filters, and, like bacteria, they are undeniably microorganisms with a limiting cell membrane and internal structure. On this basis they are considered by some to be a specialized group of bacteria which to date have been cultivated with difficulty or



at all on cell-free media. In general the rickettsiae are readily inactivated by drying, heat and antibacterial chemical agents.

**Morphology.** Rickettsiae look like very small, pleomorphic, coccoid or bacillary bacteria. They occur singly and in pairs usually, the diplobacillus

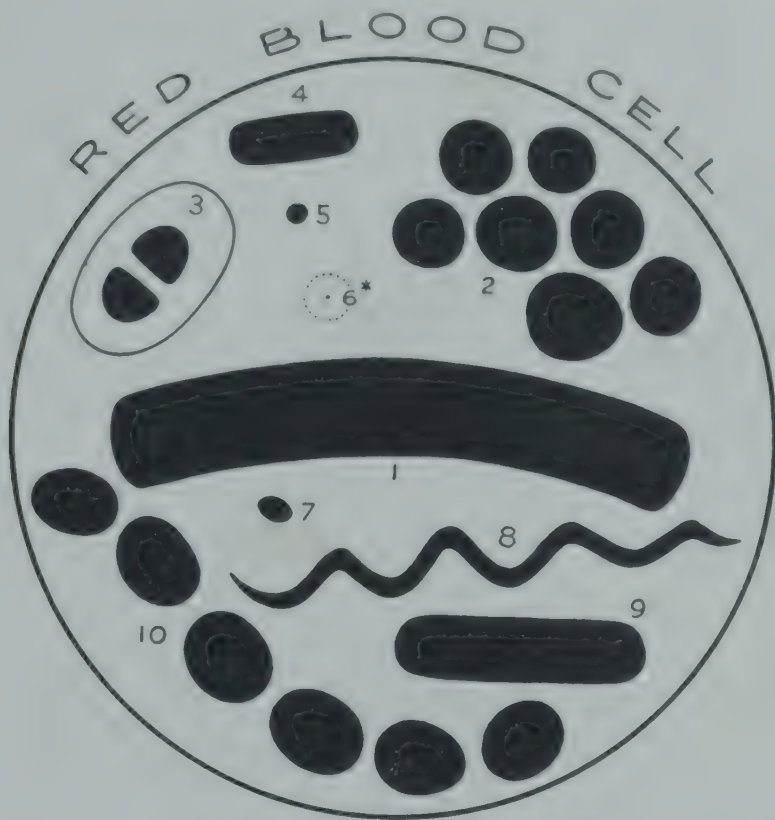


Fig. 72. Relative sizes of viruses, rickettsiae, bacteria and red blood cell. The outer circle represents a red blood cell. Within the circle:

1. *Bacillus anthracis*, 1 to 1.2  $\mu$  by 3 to 8  $\mu$ .
2. *Staphylococcus aureus*, diameter 0.7 to 0.9  $\mu$ .
3. *Diplococcus pneumoniae*, 0.8 to 1.25  $\mu$  by 1.5 to 2.5  $\mu$ .
4. *Hemophilus influenzae*, 0.3 to 0.4  $\mu$  by 1 to 1.5  $\mu$ .
5. *Vaccinia virus*, diameter 0.15  $\mu$ .
6. Yellow fever virus \* (represented by the dot within the small circle), diameter 0.018  $\mu$ .
7. *Rickettsia prowazekii*, 0.3  $\mu$  by 0.3 to 0.5  $\mu$ .
8. *Treponema pallidum*, 0.2  $\mu$  by 4 to 14  $\mu$ .
9. *Escherichia coli*, 1 to 1.2  $\mu$  by 2 to 3  $\mu$ .
10. *Streptococcus pyogenes*, 0.6  $\mu$  by 1.5  $\mu$ .

from Hopps, in Anderson, et al.: *Pathology*, C. V. Mosby Co.)

\* Approximately 3 times larger than a molecule of serum globulin.

ing the most common form, but chains of the organisms have been observed in the early stages of cell infection. Rickettsiae measure about 0.3  $\mu$  in diameter. Bacillary forms may vary in length from 0.5 to 2  $\mu$ . Their size is intermediate between that of the smallest bacteria and the largest filtrable viruses. Like bacteria, rickettsiae in tissues stain poorly with the aniline dyes, and

are usually stained by Giemsa stain or some other method ordinarily applied to spirochetes and animal cells. Bipolar staining, in which the stained ends of the cells are separated by a clear unstained area, is common. Their reaction to the Gram stain is negative. The rickettsiae are believed to be nonmotile.

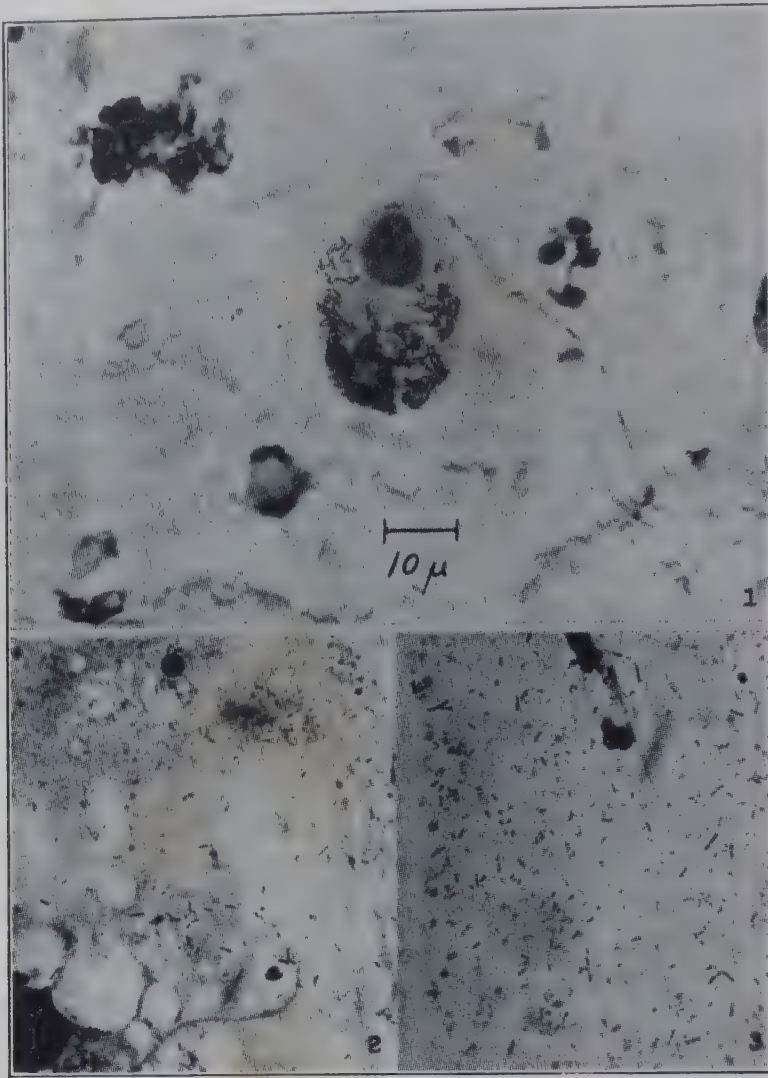


Fig. 73. Photographs of stained preparations of the rickettsiae of endemic and epidemic typhus. 1, Endemic typhus rickettsiae in the cytoplasm of an endothelial cell from an infected guinea pig; 2, smear from yolk sac of developing chick embryo infected with rickettsiae of epidemic typhus; 3, smear of yolk sac culture of rickettsiae of endemic typhus. (From Plotz, Smadel, Anderson and Chambers, *J. Exper. Med.* 77:355, 1943.)

**Cultivation.** Most rickettsiae have not been cultivated on cell-free medium. A rare exception is a nonpathogenic, extracellular rickettsia (*R. melophagi*) taken from the intestine of a blood-sucking fly. Pathogenic rickettsiae are grown in laboratory animals (for example, in the louse intestine, the anterior chamber of the rabbit's eye, the scrotum and testes of rabbits and guinea pigs), in the embryonic membranes or yolk sac of the developing chick embryo and in tissue cultures.



**Species Pathogenic for Man.** Many species of rickettsiae are found in arthropods which are nonpathogenic for man and higher animals. Species differentiation among the pathogenic rickettsiae depends upon the host naturally infected by the rickettsia, the disease it produces, the kind of arthropod that spreads the disease and the nature of the immune substances which the infected individual develops during the course of the infection. The rickettsiae which produce human disease fall into four main groups: (1) the typhus fever group,

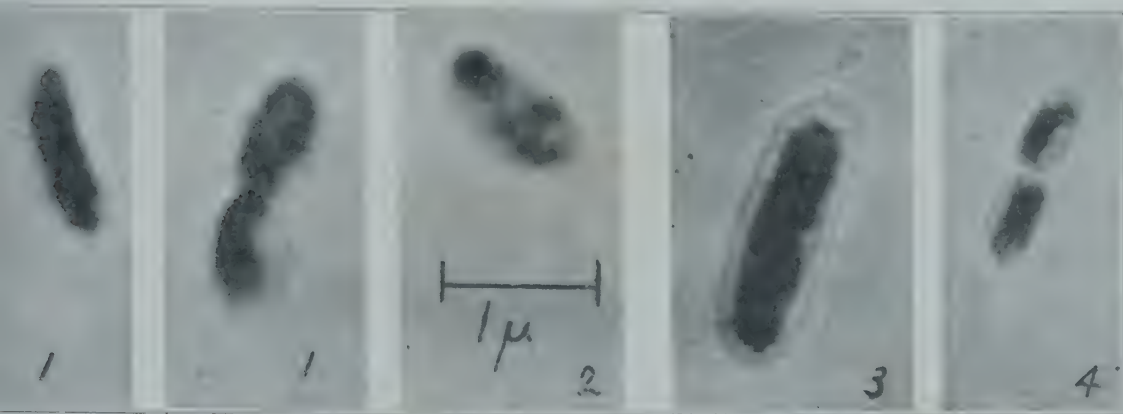


Fig. 74. Electron micrographs of the rickettsiae of (1) epidemic typhus, (2) endemic typhus, (3) Rocky Mountain spotted fever, and (4) American Q fever. (From Plotz, Adel, Anderson and Chambers: *J. Exper. Med.* 77:355, 1943.)

the spotted fever group to which the rickettsia of Rocky Mountain spotted fever belongs, (3) the tsutsugamushi group, and (4) the Q fever group as well as several newly described or incompletely known pathogens such as those of scrub fever and rickettsialpox (see Chapter 39).

More than one name has been applied to most species of pathogenic rickettsiae. The agent of Rocky Mountain spotted fever is known as *Rickettsia rickettsii* or *Dermacentor rickettsii*, the latter name deriving from a tick (*Dermacentor personi*) that transmits the disease. The rickettsia of American and Australian Q fever appears to differ from other rickettsiae in that it is filtrable and for this organism the name *Coxiella burneti* has been suggested.

## THE VIRUSES

The viruses are distinguished from usual living creatures by their inability to multiply outside of living cells, by their ultramicroscopic size and their property of passing through bacterial filters. From these properties they have been named variously **cytotropic** viruses, **ultramicroscopic** viruses and **filtrable** viruses. Their nature is incompletely known; some viruses cannot be distinguished from degraded microorganisms, and others appear to be reproducible complex protein molecules. They are recognized only by their ability to produce disease in animals and plants. If certain of the viruses are considered as degraded microorganisms, they appear to be most closely related to obligate, intracellular,

rickettsial parasites; and the bacteria, rickettsiae and viruses then form pattern of retrograde evolution from free-living organisms to degenerate forms which are entirely dependent on the metabolism of other living cells. If, however, viruses are considered as reproducible, complex molecules which from time to time are capable of establishing themselves in new host organisms, they represent a phenomenon which bridges the gap between the living and non-living world.

**Discovery of Viruses.** The history of our knowledge of the viruses begins with the development of filtration methods by which bacteria could be removed from tissue juices and culture media. Such filters, made of plaster of Paris, unglazed porcelain (Chamberland filter), certain diatomaceous earths (Berkefeld filter) and, more recently, of fritted glass, collodion and cellophane, have made possible the study of viruses as well as of bacterial exotoxins and enzymes.

The first filtrable virus was discovered in 1892 by Iwanowski who filtered juice pressed from tobacco plants which were diseased with mosaic disease and observed that the bacteria-free filtrate produced the disease in healthy plants, causing their leaves to develop mottled areas of light and dark green. The full import of this finding was not appreciated until seven years later when Beijerinck repeated and extended Iwanowski's studies and proposed his theory of a "*contagium vivum fluidum*," a living infectious fluid. In the meantime (1898) Löffler and Frosch had shown that the cause of foot-and-mouth disease of cattle was a filtrable agent, thus discovering the first animal virus. The virus etiology of yellow fever was revealed in 1901 by Reed and his coworkers. Since then the causes of many plant, animal and human diseases have proved to be filtrable agents, and it is now known that even the bacteria may be attacked by a kind of virus, the bacteriophage. The term **virus**, which formerly meant any poisonous substance or any unknown infectious agent, is now reserved specifically for this large group of filter-passing, cytotropic agents of disease. The discovery of the filtrable viruses about fifty years ago opened up a vast new field of investigation.

**Cytotropism, Inclusion Bodies and Elementary Bodies.** Perhaps the most significant single fact known about the viruses is that they multiply only inside the cells of their plant or animal hosts, some residing in the cytoplasm and others in the nucleus of the host cells. Animal viruses usually show a characteristic predilection for certain tissues and are termed **dermotropic** (skin infecting), **neurotropic** (central nervous system infecting), **viscerotropic** (internal organ infecting), and **pneumotropic** (lung infecting) according to the tissues or regions they attack. Widespread parasitism of many tissues is attained by the so-called **pantropic** viruses.

In many, but not all, virus diseases the affected cells when properly stained show **inclusion bodies** not present in normal cells. In some diseases, such as rabies, psittacosis and smallpox, these are found only in the cytoplasm; and in others, for example in yellow fever, chickenpox and poliomyelitis, they occur characteristically inside the nucleus. There is evidence that the inclusion bodies in certain lesions represent aggregations or "colonies" of the virus, but this



relationship has not been established definitely in many virus-infected tissues. At present certain inclusion bodies are regarded by some as reaction products of the host cell resulting from the presence of the virus. However, psittacosis, smallpox, vaccinia and several other intracytoplasmic inclusion bodies have been found to contain granules, termed **elementary bodies**, which have been demon-



Fig. 75. Diagram of stages in development of intracytoplasmic inclusion body in a cell infected with the psittacosis virus. a. Normal cell (n, nucleus); b. infected cell showing inclusion body (ib) in early stage of development; c. later stage showing elementary bodies (eb) within the inclusion body; d. multiplication of elementary bodies; e. release of elementary bodies from the inclusion body. (Redrawn from van Dyken and Rhodes: *Virus Diseases of Man*, 1st ed., Oxford University Press.)

strated conclusively to be the infective units of the disease. The evidence suggests that either the elementary body is the virus particle, or that the virus is closely associated with this structure.

**Size and Shape.** The ability of viruses to pass filters which retain bacteria and their invisibility under the microscope signify that they are, in general,

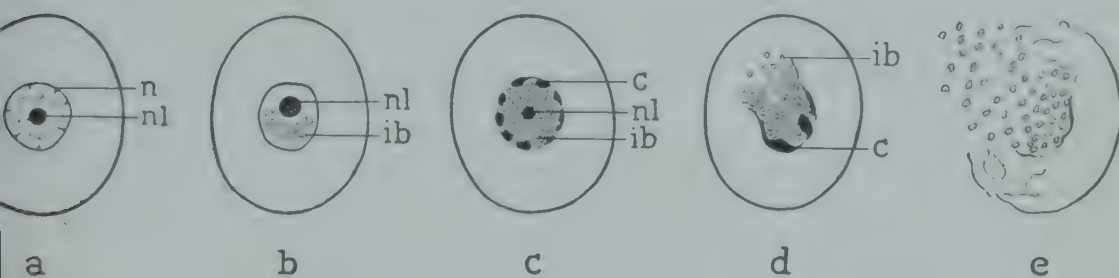


Fig. 76. Diagram of stages in the development of intranuclear inclusion bodies in a cell of animal infected with the yellow fever virus. a. Normal cell (the dark body in the nucleus (n) is the nucleolus (nl)); b. infected cell showing intranuclear inclusion bodies (ib); c. later stage in which inclusion bodies fill the nucleus and the nuclear membrane becomes plastered with chromatin (c); d and e. later terminal stages in which nucleus ruptures allowing release of the inclusion bodies. (Redrawn from Cowdry and Kitchen: *Am. J. Hyg.* 11:227, 1930.)

much smaller in size than bacteria and rickettsiae. Particles believed to be viruses have been photographed through microscopes equipped with special lenses and illuminated by ultraviolet light or by the electronic rays of the electron microscope. Some of the larger ones are visible under the highest powered lenses of the ordinary light microscope in special preparations. Viruses range in size from

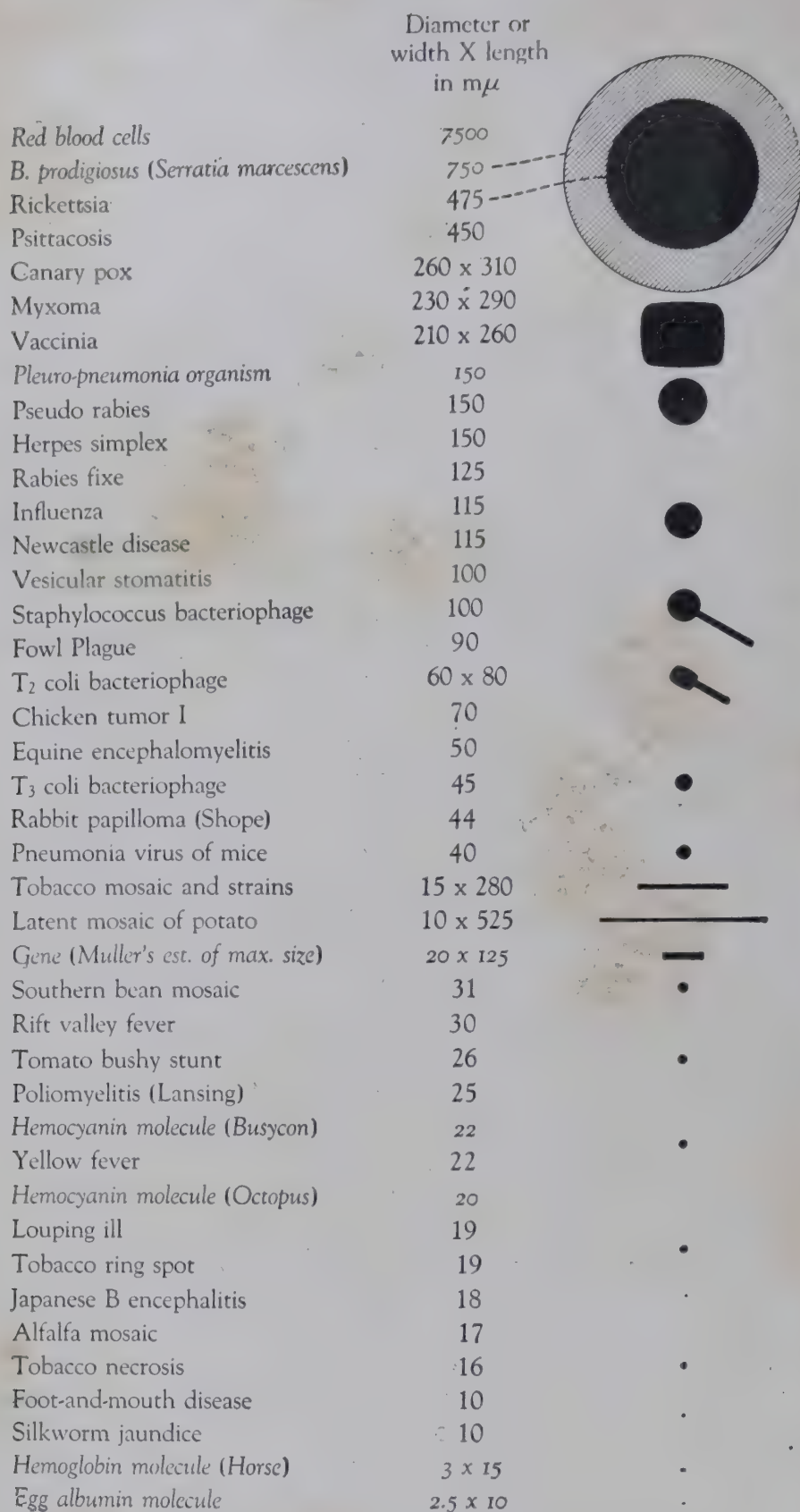


Fig. 77. Approximate sizes of viruses and reference materials. Dimensions are stated in millimicrons. One millimicron equals one-thousandth of a micron. (From W. M. Stanley: *Chemical and Engineering News*, 25:3786, 1947.)



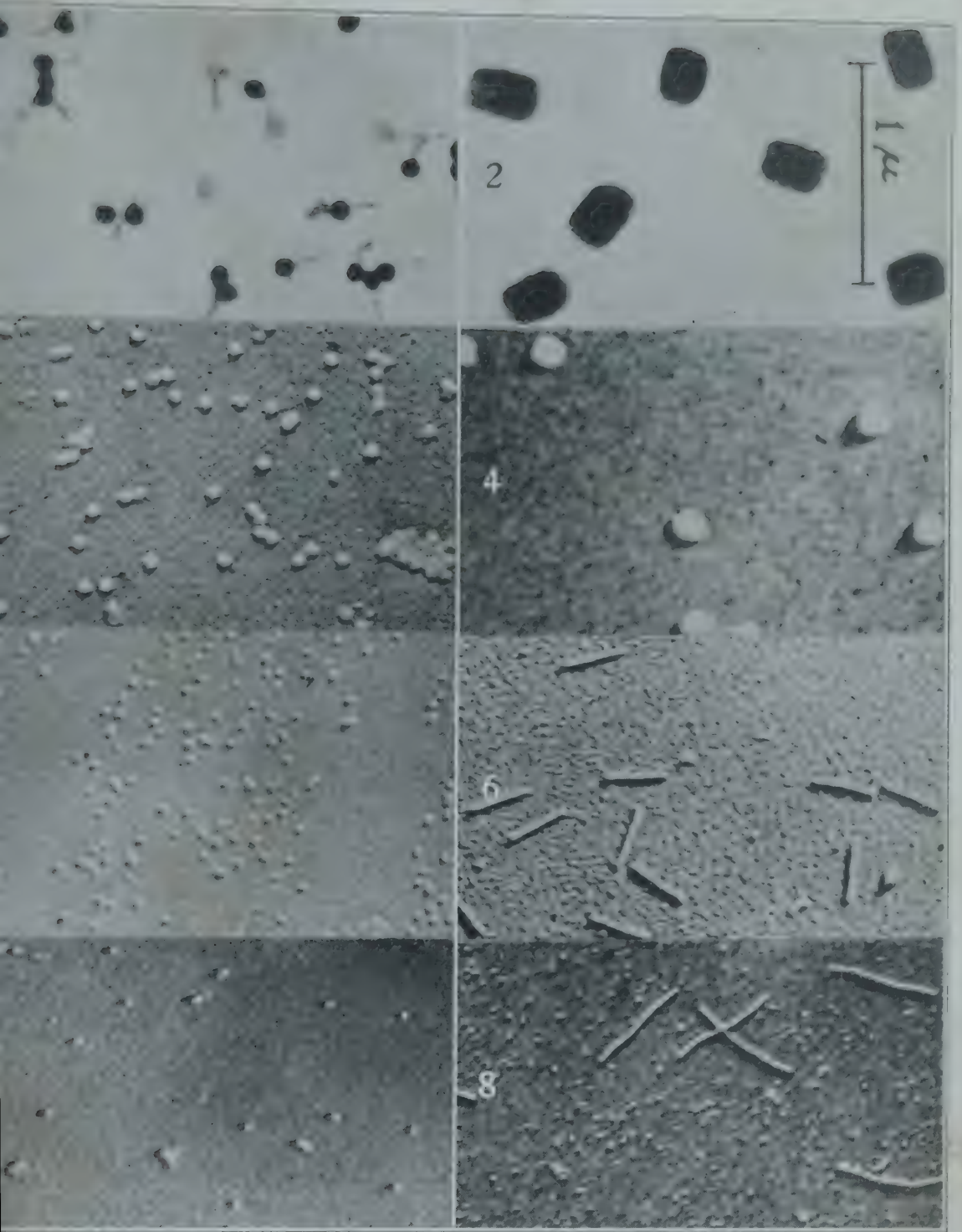


Fig. 78. Electron micrographs of some highly purified viruses. (1)  $T_2$  bacteriophage. (2) Vaccinia virus. (3) Shope papilloma virus. (4) Influenza virus (Lee strain). (5) Southern bean mosaic virus. (6) Tobacco mosaic virus. (7) Tomato bushy stunt virus. (8) Potato-X virus (latent mosaic of potato). All mounts except 1 and 2 were shadowed with gold before examination in the electron microscope. The micrograph of vaccinia virus is a reproduction of that of Green, Anderson and Smadel (*J. Exper. Med.* 51, 1942). The remaining micrographs were obtained by Drs. Oster, Sigurgeirsson, Knight and Stanley. (From Knight, C. A.: *Cold Spring Harbor Symposium on Quantitative Biology* 12:115, 1947.)

particles no larger than complex protein molecules to bodies whose dimensions approach those of the rickettsiae.

The size of viruses can be estimated by different methods: by filtration, centrifugation, diffusion and optical measurement. By filtering virus-containing fluids through collodion membranes of known and graded pore size (gradocol membranes) it is possible to determine the smallest pore through which a particular virus will pass and also the largest pore that will retain the virus. From the



Fig. 79. Crystals of the tobacco mosaic virus. ( $\times 675$ .) (From Stanley, W. M. *Am. J. Botany* 24:59, 1937.)

information the approximate diameter of the virus can be calculated. Estimation of size by means of centrifugation depends on the rate at which the virus particles are thrown out of suspension by centrifugal force, *i.e.*, their sedimentation rate at a known speed of rotation. Since ordinary centrifuges will not accomplish sedimentation of the smaller viruses, the ultracentrifuge, which revolves at speeds sufficiently great to cause the settling out of particles no larger than protein molecules, must be used. The dimensions of virus particles and some idea of their shape may be obtained by use of photography with ultraviolet light or with the electron microscope. At a magnification of around 20,000 to 50,000 diameters the images of these bodies are well defined and can be measured. Some of the larger ones are visible in special preparations under the highest powered lenses of the ordinary light microscope. Estimates of virus size made by filtration, centrifugation and photomicrographic methods are in remarkably close agreement. The chart in Figure 77 gives an idea of the size range of some of the



uses and a comparison of their diameters with those of certain bacteria, viruses and protein molecules.

Not all viruses have the same shape. Photomicrographs of the viruses of tobacco mosaic, cucumber mosaic and some other plant diseases show them to be long thin rods, while the tobacco necrosis virus and the virus of tomato yellow stunt appear to be nearly spherical particles. Certain bacterial viruses (bacteriophages) are photographed as spherical bodies, whereas others are provided with a tail-like appendage. The animal viruses that have been described to date are evidently spherical or short rectangular bodies. Elementary bodies of vaccinia, the most thoroughly studied of the animal viruses, are brick-shaped with five dense areas which appear darker than the rest of the particle. Electron micrographs have been made of nearly spherical infectious bodies which were separated from fluids and tissues of chick embryos infected with the viruses of influenza and equine encephalomyelitis. The body representing the virus of the latter disease is said to have a dense central portion. Research continues in an attempt to ascertain if the infectious body is the virus or the virus plus tissue substance added to it.

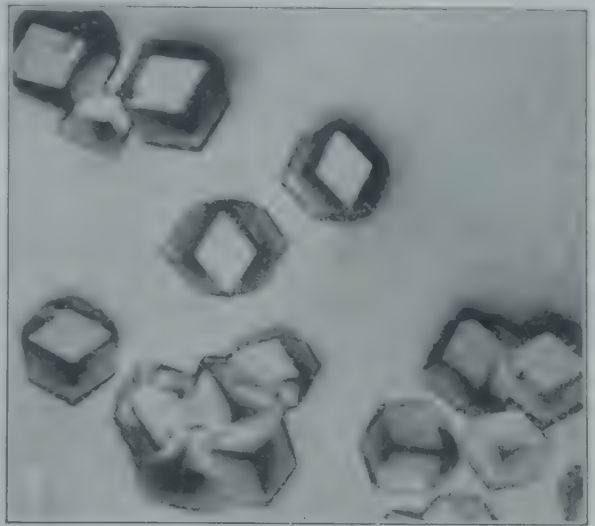


Fig. 80. Crystals of the tomato bushy stunt virus. (From Stanley, W. M.: *J. Biol. Chem.* 135:437, 1940.)

**Chemical Nature.** Chemical analyses of viruses have been hindered by lack of satisfactory methods for isolating them from the cell substance of their hosts. The first one to be isolated in a chemically pure state was the tobacco mosaic virus. In 1935 Stanley obtained from diseased tobacco plants a crystalline nucleoprotein having all the properties of the virus. Subsequent investigations confirmed the fact that this virus is a nucleoprotein which forms needle-shaped crystals and has a high molecular weight of about 40 million. Inoculated into healthy plants this protein produces tobacco mosaic disease and, on succeeding analysis, the presence of the heavy-weight protein, not found in normal plants, can be demonstrated in amounts far greater than that originally introduced. These huge "macromolecules" multiply in the plant cells. Other plant viruses have also been proved to be nucleoproteins.

As yet no animal viruses have been obtained in crystalline form, but the elementary bodies of some of the larger ones have been "purified," *i.e.*, they have been freed from host cell substances. The chemical composition of the elementary bodies of vaccinia include a high percentage of nucleoprotein, some lipid and a trace of carbohydrate. Recent analyses of the infectious particles supposed to be the virus of influenza indicate that their chemical constitution includes lipid

and carbohydrate as well as nucleoprotein; the virus of equine encephalomyelitis and certain other animal viruses contain lipid in addition to nucleoprotein. Again the question arises: "Has the animal virus been separated entirely from the host substance?" If so, present evidence is not compatible with the idea that all viruses are macromolecules of nucleoprotein, but rather that some are more complex aggregates of organic matter. It can be concluded, however, that viruses are either

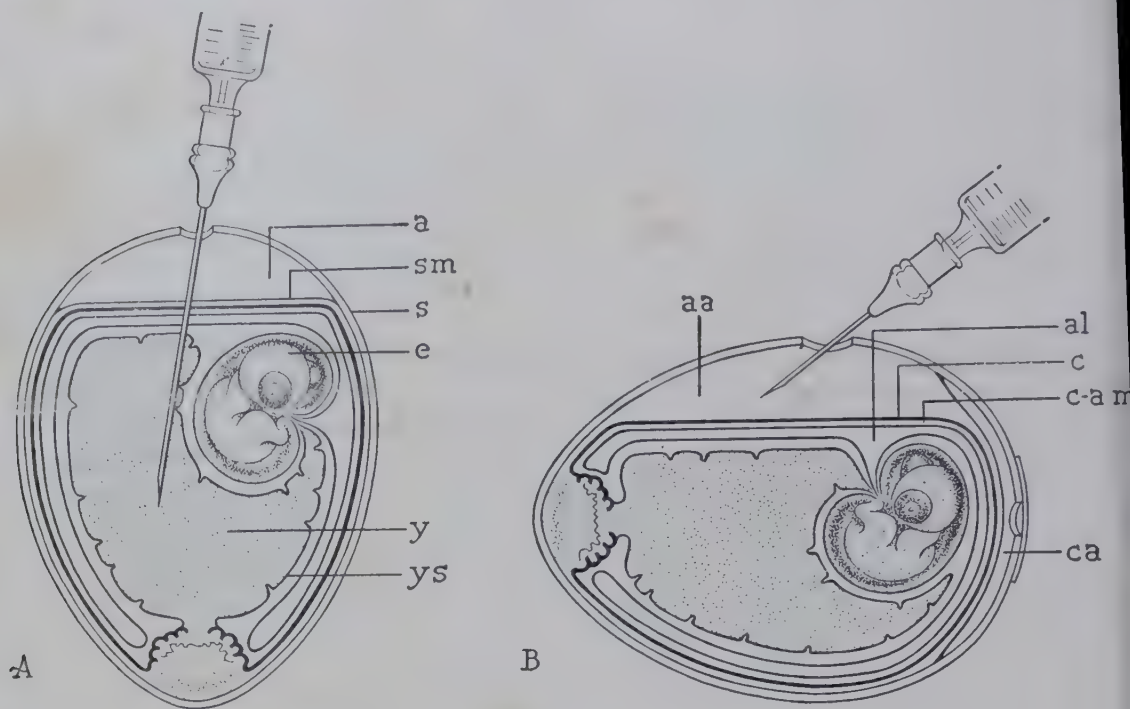


Fig. 81. Diagram of methods of inoculation of fertile eggs.

- A. Inoculation of the yolk sac, a method commonly used for the cultivation of rickettsiae and also of certain viruses. a, Air space; sm, shell membrane; s, shell; e, embryo; y, yolk; ys, yolk sac.
- B. Inoculation of the chorio-allantoic membrane, a method used for cultivating many viruses. aa, Artificial air sac; ca, collapsed air sac; al, allantoic sac; c, chorion; c-am, chorio-allantoic membrane.

(Redrawings courtesy of E. R. Squibb & Sons.)

wholly or largely nucleoprotein in composition, and that any unreserved description of their chemical as well as their physical nature must await further investigation.

**Adaptability of Viruses and Practical Aspects of Virus Mutations.** An important character of viruses is their ability to vary spontaneously under natural conditions or as an adaptive response to unusual environmental factors. When there arises a virus with properties different from those of its predecessors, the change may be irreversible, *i.e.*, the new property may be possessed by subsequent virus generations. In the language of genetics such a spontaneous irreversible change is known as a **mutation**, and the term, whether suitable or not, has been applied to permanent variations in the viruses. Virus mutations



be induced by laboratory-controlled procedures such as passage of the virus through an unnatural host or by growth in tissues not infected naturally. Of particular interest and practical value is the occurrence of new strains which differ from parent strains in their disease-producing power. Although completely unaware of the nature of the rabies agent, Pasteur observed that the

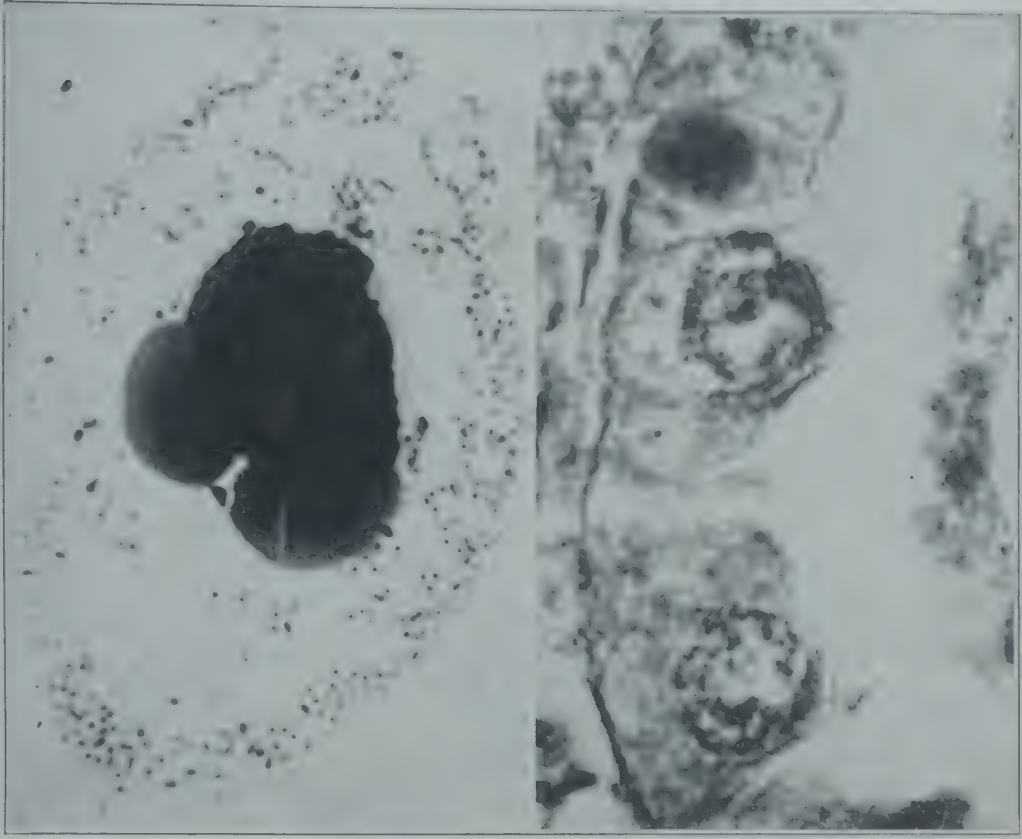


Fig. 82. (Left) Elementary bodies of a pneumonia-producing virus in a mononuclear cell (the nucleus partially covers two erythrocytes) from the lung of a mouse. (Hematoxylin stain,  $\times 2,500$ . (From Nigg and Eaton: *J. Exper. Med.* 76:497, 1944.)) (Right) Section of mouse lung infected with the same virus. The elementary bodies are shown in cells lining a small bronchiole.  $\times 2,380$ . (Courtesy of Dr. Clara Nigg.)

virulence of the "street virus" obtained from the saliva of the mad dog could be altered by repeated passage through a series of rabbit brains. Thus he developed a strain having a minimum incubation period and converted the "street virus" into the "fixed virus" which was useful in protecting an individual against the disease. An example of modern manipulation of a virus which resulted in a strain suitable for human vaccination is the laboratory-induced attenuation of the yellow fever virus. In a natural case of the disease this virus attacks the internal organs and the central nervous system. Theiler in 1930 found that on passage through mouse brains the yellow fever virus lost its viscerotropic properties. Theiler's strain was later further altered by repeated cultivation in tissue cultures, and the strain 17D emerged which is the one now used in human vaccination against yellow fever. Modified strains of the human influenza virus

have resulted from its cultivation in mice and in the chick embryo. An old example of "biological engineering" is the conversion of the human smallpox virus into vaccinia virus by growing it in calves or rabbits. Reversal of mutant strains to their original character is always a possibility, and laboratories producing vaccines of active modified viruses must be alert continuously to this contingency.

**Cultivation.** Plant viruses propagate only inside susceptible plant cells and animal viruses must be supplied with suitable animal cells if they are to multiply. Consequently techniques employed in the cultivation of animal viruses include animal inoculation and cultivation in embryonated eggs and in tissue culture. Embryonic tissues are susceptible to many viruses which will not grow in the mature tissues of the same animal. For example, many viruses which do not infect the chicken may be grown by inoculations of the extraembryonic cavities and membranes or in the chick embryo itself (Fig. 81). The inability of viruses to increase outside the living host cell and the failure of experimental attempts to detect any enzyme activity of the viruses suggest that they have no independent metabolism. The growth of viruses seems to be entirely dependent on the activity of the cells they inhabit.

**Common and Important Virus Diseases of Man.** A great many of man's most prevalent and serious infections are caused by viruses. Some of these are strictly human diseases while others are primarily animal diseases to which man is also susceptible. Veterinarians and others familiar with the diseases of animals know that there are a number of virus infections which animals do not share with man. The important economic problem of crop loss due to plant viruses can only be mentioned here. The following lists give some idea of the wide range of animal hosts attacked.

VIRUS DISEASES OF MAN	SOME VIRUS DISEASES OF ANIMALS TRANSMISSIBLE TO MAN
Smallpox	Cowpox
Vaccinia	Foot-and-mouth disease
Chickenpox	Equine encephalomyelitis
Measles	Rabies
Mumps	Psittacosis (Ornithosis)
Common cold	
Influenza	
Poliomyelitis	SOME VIRUS DISEASES OF ANIMALS NOT TRANSMISSIBLE TO MAN
Herpes	Distemper
Encephalitis lethargica	Fowlpox
Epidemic encephalitis	Myxoma of rabbits
Lymphocytic choriomeningitis	Fowl sarcoma
Trachoma	Hog cholera
Warts	
Molluscum contagiosum	
Dengue	
Pappataci fever	
Yellow fever	
Lymphogranuloma venereum	



**Bacterial Viruses (Bacteriophages).** The bacteriophage was considered one of its discoverers (Twort) to be an inanimate lytic agent like an enzyme, by the other (d'Herelle) to be an extremely small microorganism. Speculation along these lines has continued. Since a bacteriophage is a filtrable agent which multiplies only in the presence of living bacteria, it may be regarded as a virus, and the question of its nature becomes part of the problem dealing with the nature of all viruses. Since the original discoveries of Twort and d'Herelle (1915, 1917) varieties of the bacteriophage have been found regularly in the intestines of man, animals and insects as well as in feces and sewage.

**Demonstration of Phage.** The lytic and lysogenic action of bacteriophages may be demonstrated either in broth or in plate cultures of bacteria.

If a drop of phage-containing filtrate is added to a young, actively growing broth culture of a susceptible bacterium the usual turbidity of the liquid will disappear after a few hours' incubation. Under the microscope no whole or intact bacteria are found in a drop from the cleared culture. The cells which have not been lysed are weak, shrunken, abnormal "ghost" forms. If the lysed culture is filtered and a drop of the filtrate is added to another young culture of the same bacterium the second culture will be

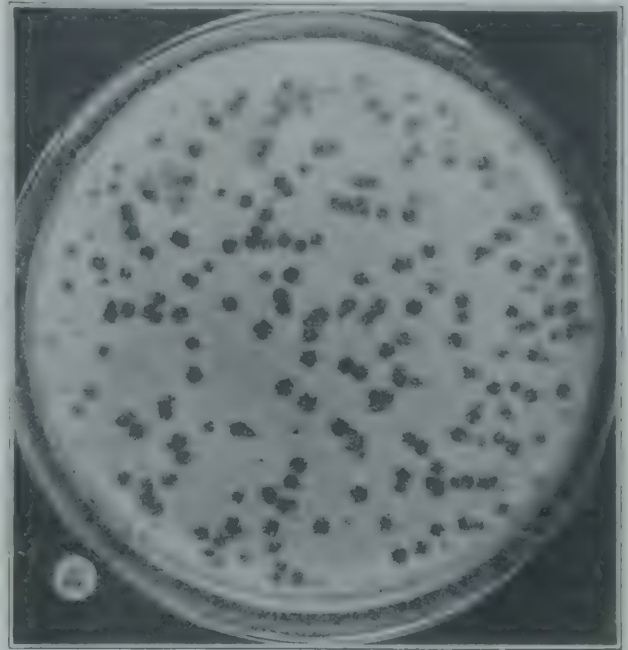


Fig. 83. Photograph of bacteriophage plaques in a poured agar plate culture. The light area represents growth of the bacterium (*Rhizobium sp.*); the dark spots (clear areas in the agar) are the plaques where the bacteriophage has destroyed the bacteria. (From Kleczkowska, J., *J. Bact.* 50:71, 1945.)

clear more quickly than the first. The phage multiplies in the presence of susceptible bacteria and its lytic action is transmissible from one culture to another. However, repeated transfer increases its power to lyse the bacteria.

Phage action can also be shown by inoculating the surface of an agar plate with a drop of phage filtrate and a drop of a young culture of susceptible bacteria. After incubation, clear barren spots called **plaques** are seen in the solid film of bacterial growth, and if isolated colonies are present their margins appear irregular or "moth-eaten." By substituting serial dilutions of the phage filtrate in the inoculation of such plates, it is possible to show that the higher the dilution, the fewer plaques develop. The plaque is considered to be a colony of the bacteriophage and evidence of the particulate nature of the phage. Since, theoretically, each plaque originates from at least one phage particle it is possible

to estimate how many such particles are present in a given amount of filtrate, *i.e.*, to determine the concentration of phage in a filtrate.

Optical evidence supports the idea that phages are particulate and offers some clues as to how they attack bacteria. Examinations of sterile phage filtrates by ultraviolet and electron microscopy, as well as by direct observation and micrographs made through a darkfield microscope, show fairly homogeneous particles whose magnitude coincides with the size range of other viruses. These are generally spherical to oval bodies though in certain varieties of phage there appears to be a tail-like appendage attached to the head portion. Chemical analysis of purified phage suggests that, like the other viruses, it is largely if not entirely nucleoprotein.

**Action of Phage on Bacterial Cells.** Just how the bacteriophage attacks bacteria is not clear. From present information there is little to explain the nature of the chemical reactions or of the enzymes which may be involved. On the other hand, certain physical changes have been observed repeatedly in a mixture of bacteriophage and susceptible bacteria. The phage particles are adsorbed to the surface of the bacterial cell. A single phage particle penetrates the cell and apparently renders it refractory to subsequent penetration by other similar phage particles. After contact there is a surge of activity within the bacterium. The cell usually swells or blows bubbles out through its wall, and in a matter of minutes the cell bursts, releasing numerous new phage particles.

Thus infection of a bacterium by a single bacteriophage results in multiplication of the phage and destruction by lysis of the host cell. Whether phage

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Fig. 84. Electron micrographs showing the action of bacteriophage on bacterial cells. These preparations were made according to a technique (Hillier, J.; Knaysi, G., and Baker, R. F.: *J. Bact.*, 56:569, 1948) by which the bacteria are grown on the upper side of a very thin collodion membrane deposited on the surface of an agar plate. At time of examination the membrane is floated off the agar (with the bacterial cells on the upper side not in contact with the water), rapidly dried on a wire screen and inserted in the electron microscope. (From Hillier, J.; Mudd, S., and Smith, A. G.: *Rockefeller Laboratories, Princeton, N. J., and University of Pennsylvania School of Medicine, Philadelphia.*)

(Upper left) A microcolony of *Escherichia coli*. Normal cells. Three-hour growth at 37° C. (× 4,560.) Note internal structure of cells. (From *J. Bact.* 57:319, 1949.)

(Upper right) A similar colony of *Esch. coli* grown two hours, then inoculated with a suspension of *coli* (T<sub>2</sub>) bacteriophage so diluted that one phage particle per colony was applied. The preparation, examined 30 minutes after application of phage, shows a single lysed cell among apparently normal cells. Phage particles are visible in the remains of the cell. (× 8,550.)

(Lower left) A similar colony to that shown at upper right but examined 18 hours later. Large numbers of phage particles are visible as well as what appear to be normal cells. Examinations of such preparations give no evidence of division of phage particles. (× 7,850.) (From *J. Bact.* 56:569, 1948.)

(Lower right) A cell of *Esch. coli* packed with many hundred phage particles. Eighteen-hour preparation. (× 41,600.)



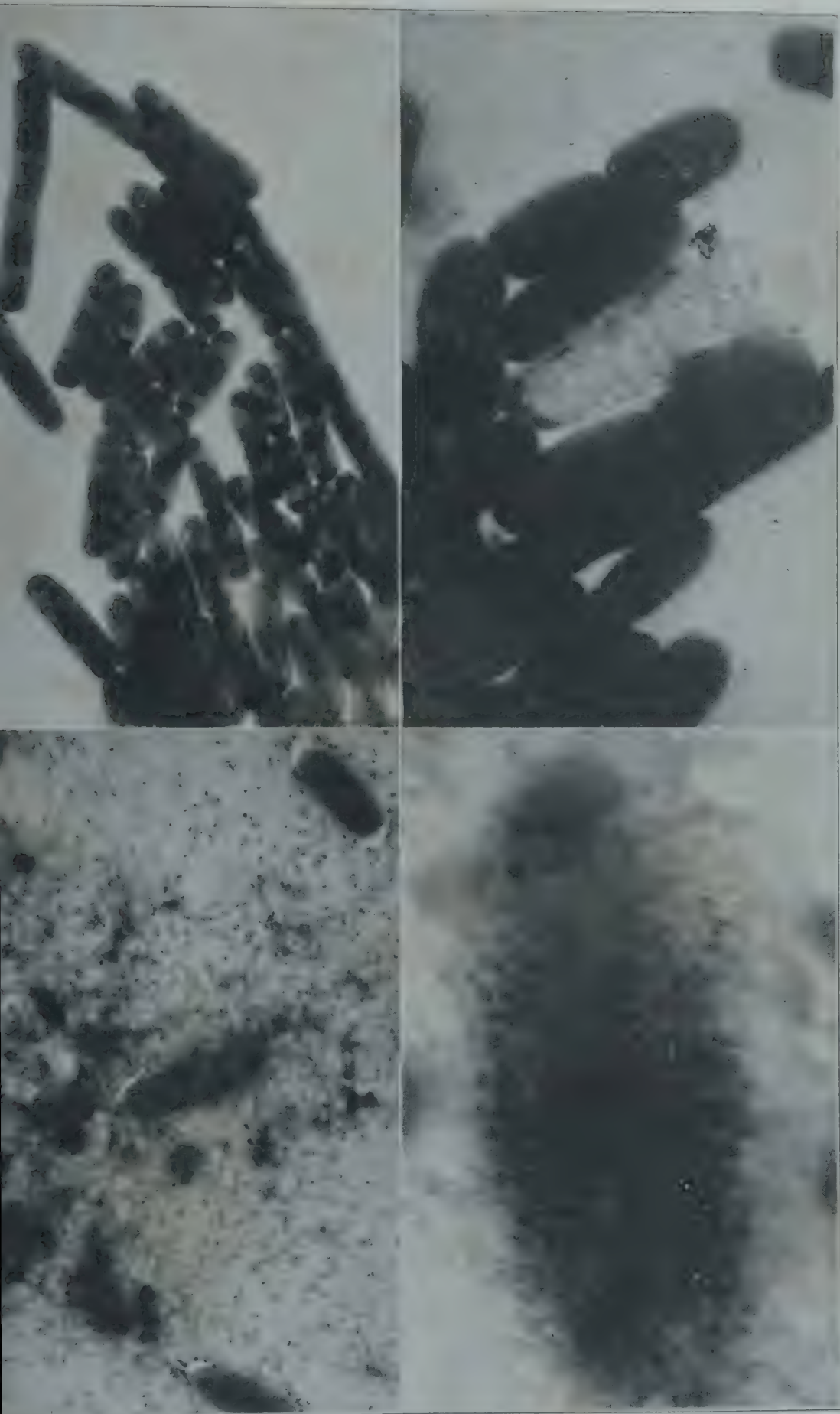


Fig. 84. Caption on page 110.

particles multiply by division, analogous to the fission of microorganisms, whether the presence of the original phage particle in the bacterial cell condition the bacterial metabolism so that the cell itself produces more of the same kind of particle is not entirely clear. Improved techniques of electron microscopy have recently provided spectacular photographic records of bacteriophage action and give evidence that the phage particles do not divide (Fig. 83). The bacteriophages are specific in their action, one kind of phage lysing only one strain of bacteria or a group of closely related bacteria.

**Practical Applications of Phage Action.** The discovery of the bacteriophage aroused great hopes for its use in the treatment of bacterial infections. If the proper phage were introduced into the infected body would it not destroy the bacterial parasites and institute recovery? Unfortunately, compared to the original expectations, results of phage therapy have been disappointing. Its therapeutic use has been tested more extensively in staphylococcic infections and in bacillary dysentery than in other diseases. While hope of its value has not been abandoned by many workers, persistent trial of bacteriophage therapy continues in some quarters.

Practical application of the high degree of specificity exhibited by some varieties of phage has been made in another field, namely in the identification or **phage typing** of certain strains of typhoid bacilli, dysentery bacilli, staphylococci and hemolytic streptococci.



## Part 2

# Methods for Studying Microorganisms

## 9

### THE MICROSCOPE AND MICROSCOPIC METHODS

**Development of the Microscope.** There is no record of the first grinding lenses, but the art of making magnifying glasses was known to the ancient Greeks and Romans. In the thirteenth century Roger Bacon (1214-1297) discovered the principles that established the science of optics. He worked with



Fig. 85. Leeuwenhoek's microscope. (From *Antony van Leeuwenhoek and His "Little Worlds,"* by C. Dobell, 1932. Reproduced with the permission of the publishers, Court, Brace & Co., New York. In Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Appleton-Century-Crofts, Inc.)

simple magnifying lenses and suggested their use in spectacles. Invention of the compound microscope about 1600 is generally accredited to Hans and Zacharias Jansen, sons of a Dutch spectacle maker. This instrument differed from the single-lensed, simple microscope in that it combined the magnifying

powers of several lenses in one instrument. Galileo (1564–1642) developed the telescope on the same principle of lenses in series, and, although his exact contribution to the compound microscope is unknown, he also fashioned these instruments. In 1665 Robert Hooke first described the cells he observed in plant

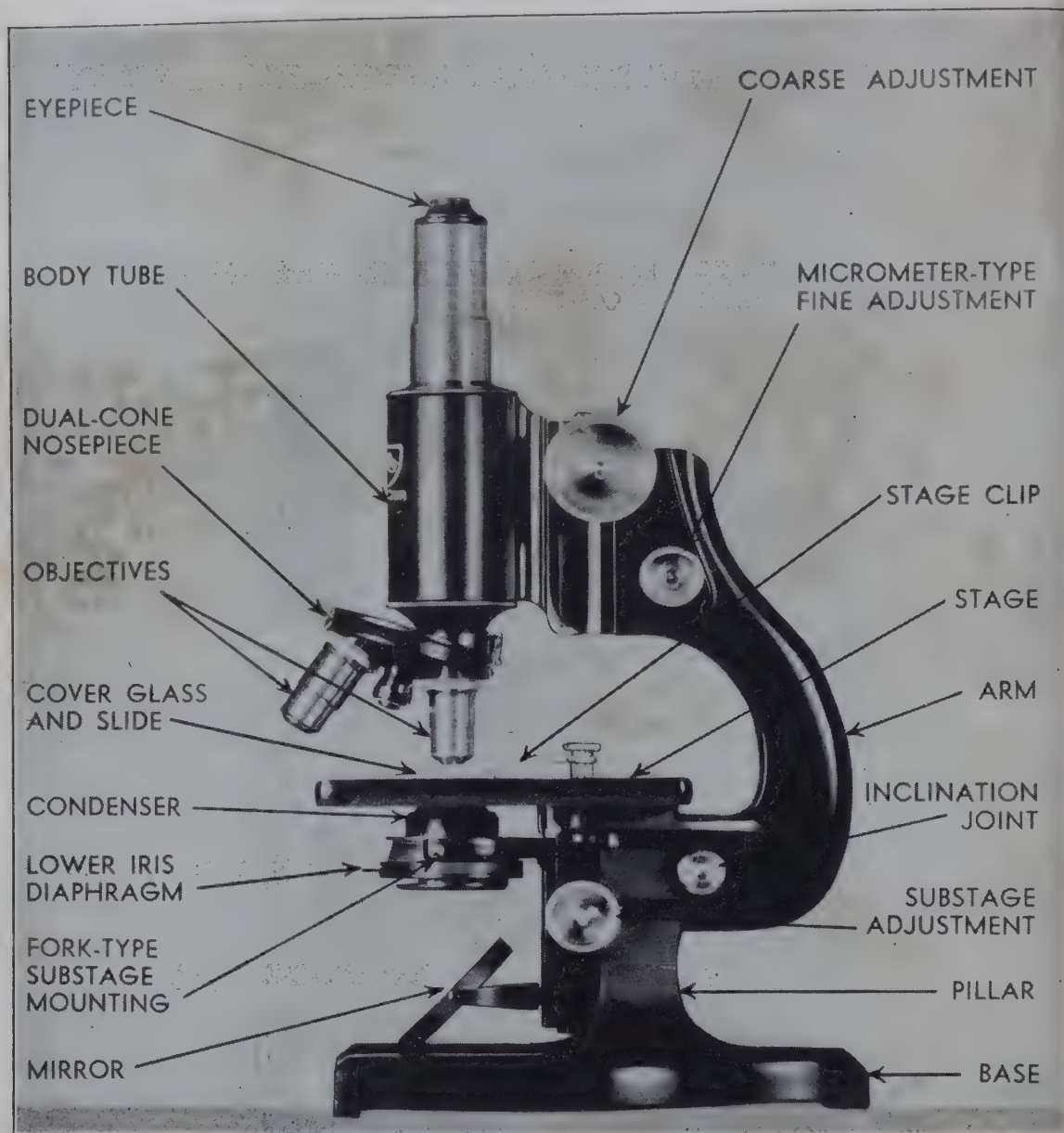


Fig. 86. Mechanical features of the compound microscope. (Courtesy of American Optical Co., Scientific Instrument Division.)

and animal tissues examined under the compound microscope. By the middle of the seventeenth century opticians were offering these microscopes for sale in the larger cities of Europe.

Application of the simple microscope to microbiology was first made by Antony van Leeuwenhoek. Investigators claim that the highest magnification he could have obtained with a single lens was about 270 diameters. Yet in 1676 he discovered and accurately described bacteria, microorganisms so small that they



the beginning student's powers of observation even when they are magnified most one thousand times by the modern compound microscope.

Following these early investigations, improvements in materials and methods of lens grinding and the combination of lenses to give sharper focus as well as greater magnification have continued to increase the value of the microscope. Early distortion of the image due to spherical and chromatic aberration caused by differences in refraction of rays of the visible light spectrum were largely overcome; the oil immersion lens which allows greater magnification and the stage condenser which concentrates the light rays on the object were developed. The basic plan of the modern compound microscope was complete.



Fig. 87. Sectional view of achromatic objectives of the compound microscope. (left) Low dry objective,  $\times 10$  (16 mm.); (center) high dry objective,  $\times 43$  (4 mm.); (right) oil immersion objective,  $\times 97$  (1.8 mm.). Compare the shapes, number and positions of the lenses in these objectives. (Courtesy of Bausch & Lomb Optical Co.)

However, by 1880. Since that time the darkfield microscope (1903), ultraviolet illumination (1925) and, most recently, the electron microscope (1940) have been introduced, each one magnifying more than the last. By means of photographic enlargements combined with the electron microscope, magnifications over 50,000 diameters have been attained.

**Parts of the Microscope Regulating Magnification.** Since the compound microscope is used to observe many sizes of objects in microbiology and other sciences the value of each instrument is increased by the range of its magnifying powers. The magnification of a microscope can be varied by using different objectives and different oculars, the optical parts in which the magnifying lenses are mounted.

The objectives commonly used in microbiology are known as the **low dry**, **high dry** and **oil immersion** objectives. They are usually marked 16 mm., 4 mm., and 1.9 mm. (or 1.8 mm.) respectively, or these figures may be expressed in terms of inches in which case the fractions  $\frac{2}{3}$ ,  $\frac{1}{6}$ , and  $\frac{1}{12}$  appear on the objectives. These numbers refer to the equivocal focal distance, and are related to the distance between the front lens in the tip of each objective and the object when it is in focus. The actual focal distance of the low dry objective, however, is not 16 mm., but the size of the real image produced by this objective is the same as that produced by a simple, single lens whose principal focal distance is

16 mm. It will be noted that the higher the magnification, the shorter the focal distance. Another number which appears on the objective states the numerical aperture (N.A.) of that objective. The numerical aperture is a measure of the resolving power of the objective, and the higher the N.A. the greater is the power

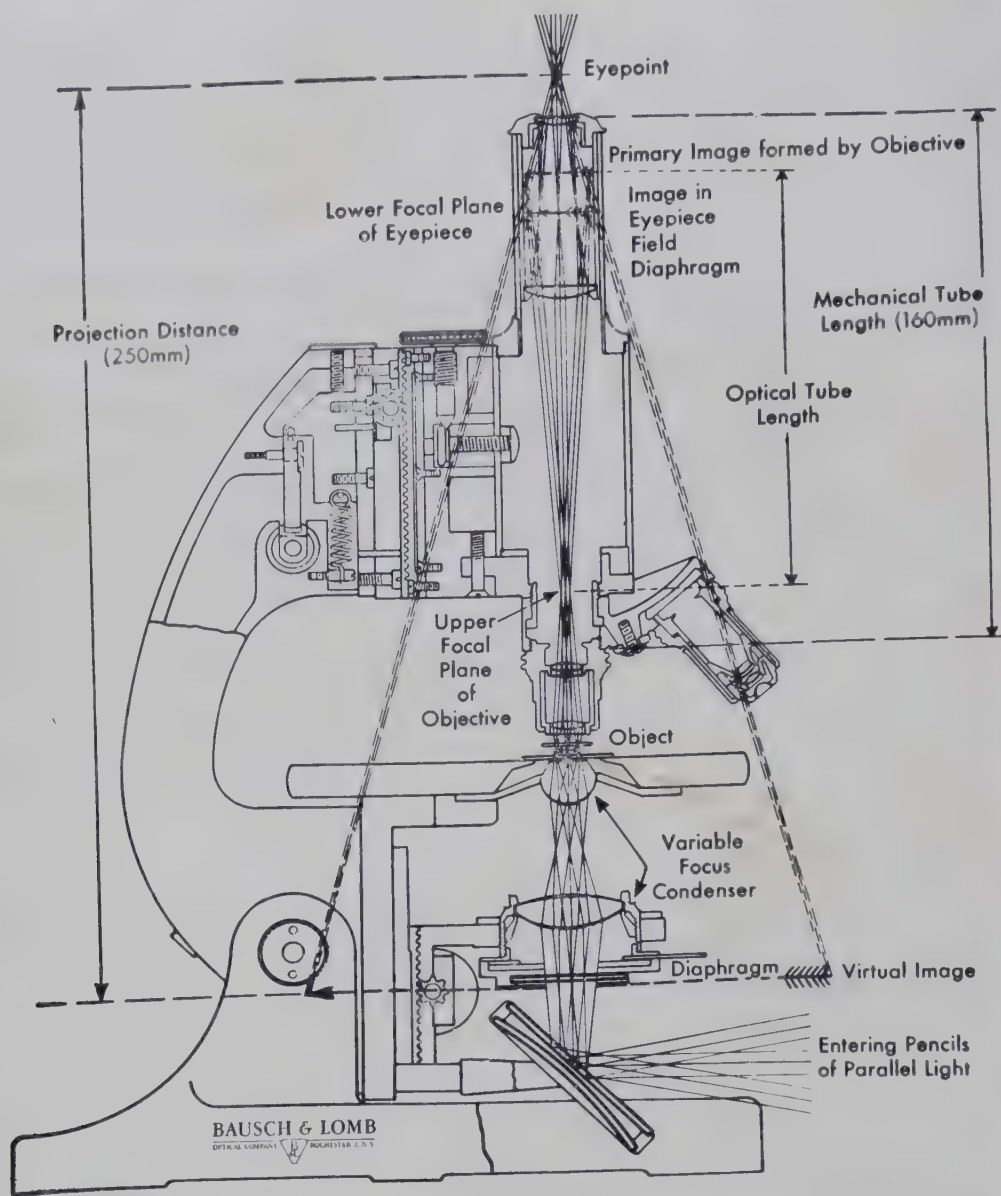


Fig. 88. Path of light through the compound microscope. (Courtesy of Bausch & Lomb Optical Co.)

of the objective to reveal fine details of structure. Each objective apart from the rest of the microscope gives a certain magnification which is often indicated on its side:  $10\times$  for the low dry, generally  $43\times$  or  $45\times$  for the high dry, and  $95\times$  or  $97\times$  for the oil immersion objective. The lenses in the low and high dry objectives must be kept dry; the light rays must pass through air between the glass slide or coverslip and their lenses, and not through water or some other liquid. On the other hand, the oil immersion lens is designed to be immersed in an oil whose refractive index is the same as that of glass, *i.e.*, the oil and glass



st bend the light rays to the same degree as they pass through them. Figure illustrates how a suitable immersion oil prevents the scattering of light rays after they have passed through the glass slide and thus makes for better illumination and definition of the magnified object.

One ocular or eyepiece fits into the top of the draw tube of the monocular microscope through which the observer looks with one eye, while a binocular microscope has two eyepieces to accommodate both eyes. The function of the

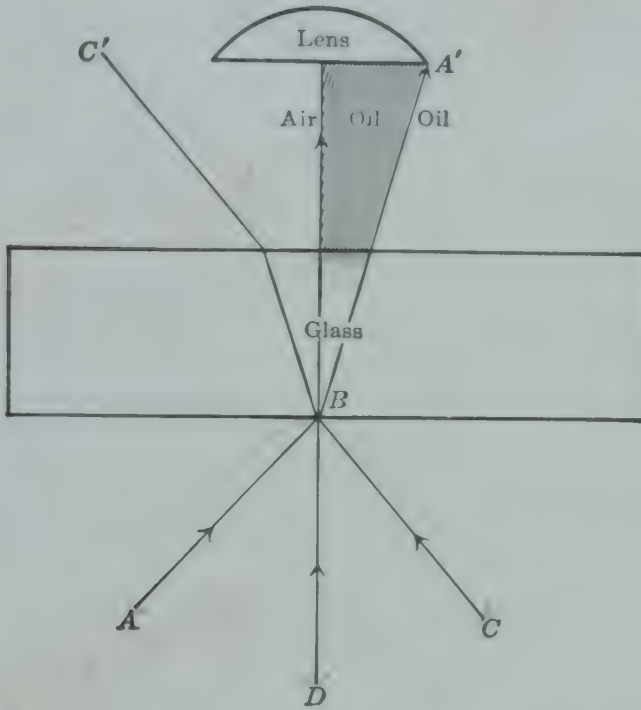


Fig. 80. Diagram illustrating the principle of oil immersion. The ray of light  $CBC'$  is bent as it passes from glass into air, while  $ABA'$ , passing into an oil which has approximately the same refractive index as glass, continues in a straight path. (From Ward: *A Textbook of Bacteriology and its Applications*, Ginn & Co.)

ses in an ocular is to magnify the image produced by the objective. The ocular primarily employed in microbiology magnifies ten times as indicated by the  $10\times$  stamped on its side or top, but it may be replaced by one of lesser or greater magnification. The **total magnification** of an object viewed through a compound microscope, therefore, is the magnification of the objective multiplied by the magnification of the ocular. Thus the image seen when looking through a  $10\times$  ocular and the low dry ( $10\times$ ) objective is one hundred times the actual size of the object; a combination of the  $10\times$  ocular and high dry ( $45\times$ ) objective gives a magnification of  $450\times$ ; the total magnification of the  $10\times$  ocular and oil immersion ( $97\times$ ) objective is  $970\times$ .

The draw tube or body tube is the hollow cylinder separating the objective and the ocular. As its name implies, it may be pulled out or shortened to change the distance between the objective and the ocular, i.e., to vary the tube length. Each microscope functions best at a certain tube length. Drawn out

beyond this length the magnification is increased but the image becomes blurred. Most of the new microscopes have their body tubes permanently fixed at the proper tube length. In work which demands a known magnification, for instance in counting the number of bacteria in milk by direct microscopic examination, or in measuring the size of a microorganism, the length of the draw tube must be considered as well as the ocular and objective used.

**Parts of the Microscope Regulating Light.** Proper illumination of the object to be examined is of paramount importance. The amount of light ideal for stained, fixed preparations of bacteria is unsuitable for the study of living, unstained microorganisms because bright light passes through minute bodies

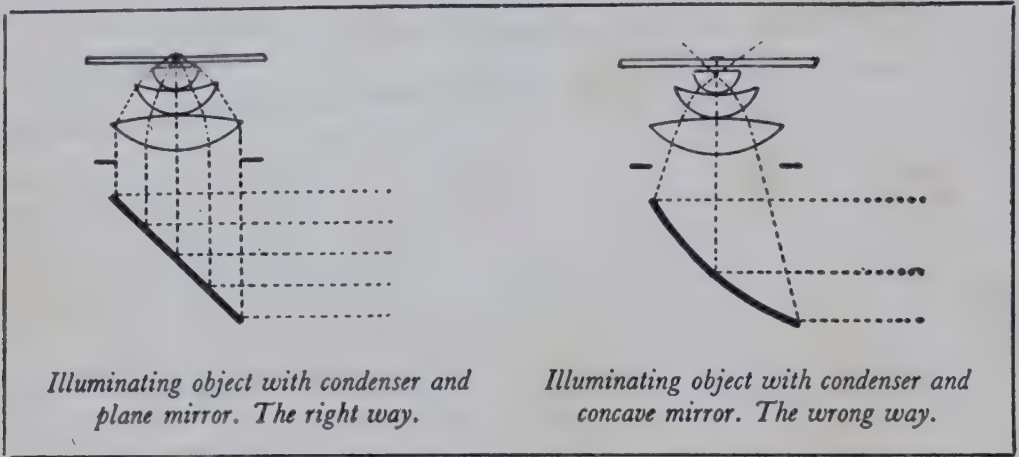


Fig. 90. Diagram illustrating use of plane and concave mirrors with the substage condenser. (Courtesy of Bausch & Lomb Optical Co.)

of transparent protoplasm and no image is seen. In liquid unstained mounts it is, therefore, generally necessary to reduce the light. Ideal illumination varies, depending on different factors such as the magnification required and the density of the object. The skilled microscopist always starts with maximum light and then experiments by reducing it until a distinct outline of the image appears against a background that is pleasant for the eyes. These parts of the microscope deal with the regulation of light: the plane and concave mirrors, the substage condenser and the iris diaphragm.

The mirrors are used to catch the light rays and to reflect them up toward the stage. Each microscope has two mirrors in a single frame, one with a plane surface and the other with a concave surface. Which of these mirrors should be used depends upon the objective employed and whether or not the light passes through a substage condenser. When stained, fixed films of bacteria are examined, the substage condenser, the oil immersion objective and the plane mirror should be used. With objectives of lower magnification the concave mirror may be used, but in bacteriology it is best to adhere to the rule of using the plane mirror with the substage condenser (Fig. 90).

The substage condenser, usually an Abbé condenser, is a device consisting of two or more lenses whose function is to bend the parallel light rays reflected by



plane mirror to a focus on the object. The rays converge to a point just above the condenser when the plane mirror is used and, therefore, when the condenser is raised flush with the stage this point of focus will be at the level of the slide. If the condenser is lowered, maximum illumination of the slide cannot be obtained.

The substage diaphragm is a circular shutter placed just below the substage condenser. Its purpose is to regulate the amount of light passing through the condenser. This is accomplished by adjusting the size of the opening in the diaphragm by a lever. When starting a microscopic examination the diaphragm should be wide open and maximum light centered on the object. Thereafter the amount may be diminished by reducing the opening in the diaphragm.

**The Coarse and Fine Adjustments.** Each microscope has two adjustments which raise and lower the body tube in focusing, *i.e.*, in bringing the lenses to a distance from the object which is optimum for the production of a clear image. The coarse adjustment works on a coarse-toothed rack and pinion, and a slight turn of the screw changes the distance between the objective and the stage appreciably. The coarse adjustment should not be turned away from the operator, *i.e.*, never be used to focus down, while looking into the eyepiece, for fear of striking the slide with the front lens of the objective. The fine adjustment works on the same principle as the coarse adjustment, but is designed to raise or lower the body tube very gradually. Used properly to clear up the image and to find it, the fine adjustment need not be turned more than half a revolution in either direction.

**Supporting Structures of the Microscope.** From the labelled illustration (fig. 86) the parts of the microscope which support the optical parts and slide preparations are evident. The base should be directly in front of the worker and conveniently close to the edge of the table. The inclination joint allows the upper part of the microscope to be tilted, but this is not necessary for comfort if the microscope is properly placed and the worker sits close to it. The instrument is lifted from one place to another by its arm. The stage may be equipped with a mechanism for holding and moving the slide, a so-called mechanical stage. The revolving eyepiece snaps the objectives into place directly in line with the body tube.

**Care and Use of the Microscope.** The microscope is a delicate instrument which must be kept spotlessly clean and handled carefully. Through the microscope a spot of grease on the eyepiece or a bit of oil or dust on the lens of the objective will appear many times enlarged and will interrupt the image of the object. Therefore one must be sure that the lenses and mirrors are clean before each microscopic examination. The exposed lenses should be wiped thoroughly with clean lens paper and no other material. If the tip of the objective is sticky or covered with dirt that ordinary wiping will not remove, it should be cleansed with lens paper moistened with xylene and polished with a dry piece of lens paper. The objectives and eyepiece should not be taken apart or removed from the microscope ordinarily, for as long as they remain in place there is no way for dust

to reach the inner parts. The exposed lenses should not be touched by fingers nor should the eyelashes contact the eyepiece.

At the start of a microscopic examination the body tube should be raised to allow freedom of motion in placing the slide on the stage. Before focusing, the diaphragm should be wide open, the position of the substage condenser should be checked and the mirror adjusted to throw a bright spot of light on the condenser lens. The desired objective is then swung into line with the ocular. If the

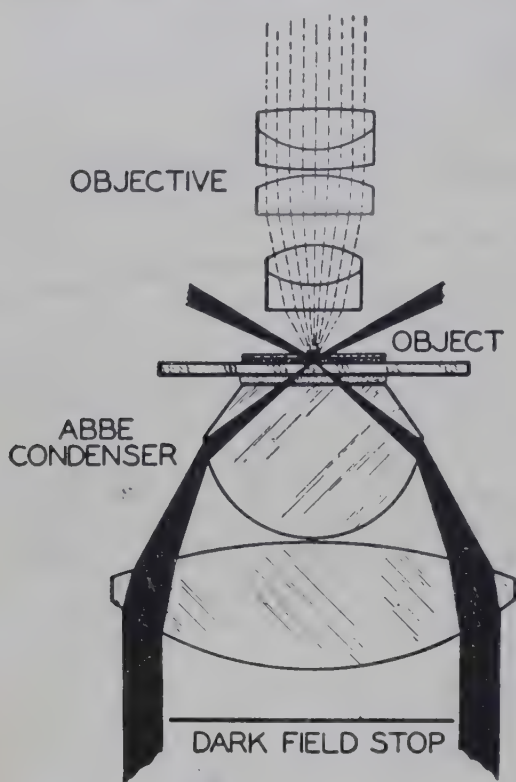


Fig. 91. Darkfield illumination using the Abbé condenser with a dark-field stop. (Courtesy of Bausch & Lomb Optical Co.)

oil immersion objective is used, a drop of oil should be placed on the slide in the center of the preparation. Next, with eyes on the objective (not at the eyepiece), the operator turns the coarse adjustment to lower the body tube until the tip of the objective almost touches the cover slip or slide. Looking into the eyepiece, the adjustment of the mirror is then tested for maximum light which later may be reduced by the diaphragm, and the body tube is raised gradually by turning the coarse adjustment until the image is located. While focusing, the slide should be held down firmly by clips, the clamps of a mechanical stage, or by hand. The oil immersion objective is immersed in the oil when the object is in focus.

If the first try fails to find the object, the entire focusing procedure must be repeated, starting with the lowering of the objective until, viewed from the side, its front lens almost touches the slide or coverslip. Once the object is in focus the

slide should be moved about to observe several fields. Constant focusing with the fine adjustment is necessary when using high magnification and especially when moving to a new field on the slide. On completion of the study the body tube should be raised before removing the slide from the stage. The routine of using the microscope is not complete until the oil has been wiped from the oil immersion objective and the stage, as well as all other parts, is perfectly clean.

**Darkfield Microscope.** Particles too small or not dense enough to be seen in a well lighted field are often visible by reflected light against a dark background. This principle is illustrated by the visibility of dust particles that are seen when a shaft of bright sunlight penetrates an otherwise darkened room. A **darkfield** or **ultramicroscope** reproduces this effect. To convert a bright field microscope into this type, a special darkfield condenser must be substituted for the regular substage condenser, or the top lens of the ordinary condenser may



replaced by a lens which blocks all vertical rays and allows only the most oblique rays to illuminate the slide. A special oil immersion objective may be used, or a device is inserted into an ordinary oil immersion objective to reduce its aperture. Strong light is required and the space between the condenser lens and the slide, as well as between the slide and the tip of the objective, must be filled with immersion oil.

By these arrangements the light does not pass straight up through the slide, but the oblique, almost horizontal, rays are admitted from the sides and centered on a point of focus on the object causing bacteria and other microscopic particles to appear bright against the dark background. Only liquid mounts made with special media and coverslips of certain thickness are adaptable to darkfield illumination. This type of examination is especially valuable in the study of spirochetes.

**Phase Microscope.** Phase microscopy was developed for the purpose of revealing transparent structures of living, unstained microorganisms and tissues which can be seen with difficulty or not at all under the ordinary brightfield microscope. If a structure differs from the substance surrounding it in thickness or index of refraction for light, *i.e.*, in optical path, it is made visible by the phase microscope. Invisible changes in the phase of the light due to optical path differences are transformed to differences

in light intensity which are visible and may be photographed. Contrast in the phase microscope is controlled by a diffraction plate which is introduced into each objective of this microscope. Different types of diffraction plates allow particles of higher refractive index and/or greater thickness to appear brighter (bright contrast) or darker (dark contrast) than the background, and regulate the degree of contrast between the particle and its background. An ordinary light microscope can be converted into a phase microscope by replacing the usual condenser and objectives with a phase condenser bearing an annular diaphragm and phase objectives equipped with diffraction plates.

**Fluorescence Microscope.** The use of fluorescence microscopy for the diagnosis of *Mycobacterium tuberculosis* is based on the fact that acid-fast organisms stained with auramine O, a fluorescent dye, and subsequently treated with a suitable decolorizing solution, retain the fluorescent dye whereas other organisms and the surrounding material in the preparation are decolorized. The dye absorbs invisible ultraviolet radiation and emits it as a yellow light. An ordinary microscope can be equipped as an ultraviolet light microscope by re-



Fig. 92. A spirochete (*Treponema pallidum*), red blood corpuscle and leucocyte under darkfield illumination. (From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Appleton-Century-Crofts, Inc.)

placing the silver mirror with an aluminized mirror and inserting a yellow filter into the eyepiece below the top lens. An 8 mm. objective and a  $20\times$  eyepiece is recommended for this examination. A blue, ultraviolet-transmitting filter is placed over a low-voltage, high-amperage concentrated filament type of microscope

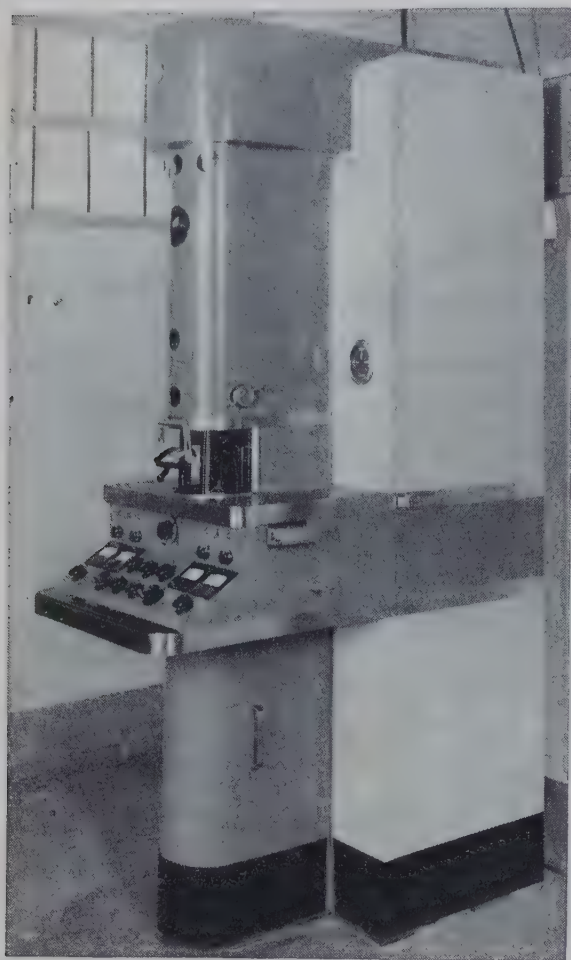


Fig. 03. The RCA electron microscope, type EMU. Magnification between  $1000\times$  and  $20,000\times$  on the fluorescent screen, which makes the final image visible to the person operating the microscope, or on a photographic plate which makes a permanent record of the image. The photograph may be magnified by photographic enlargement up to 100,000 diameters. (Courtesy of the Radio Corporation of America.)

greater the power of resolution and the higher the useful magnification obtained. Visible light as used in ordinary illumination has a wave length of about 5000 Ångström units\* ( $0.5\ \mu$ ). Rays of ultraviolet light vary in wave length from 4000 Ångström units to a lower limit generally accepted as about 1000 Å. If instead of visible light, the shorter waves of invisible, ultraviolet light illuminate

lamp. The examination should be made in a darkened room or the microscope should be shielded from light. Proper illumination reveals luminous, yellow bacilli against a black background.

**Limitations of Light Microscopes.** Even the best light microscopes have a limit of useful magnification beyond which further structure of the object is not revealed, but the object is only further enlarged. The ability to discern structure depends on the resolving power of the microscope or its power to form distinct images of minute structures which are separated from each other by a very short distance. The resolving power of the human eye is about 0.1 millimeter, *i.e.*, the unaided eye can distinguish between two points or lines if they are not less than about 0.1 mm. apart. If they lie closer together than this distance, they will blur together and be seen as one point or one line. The resolving power of a compound microscope with an ideal lens system is about  $0.2\ \mu$  ( $0.0002\ \text{mm.}$ ) and its maximum useful magnification is approximately 1100 diameters. The limitations of light microscopes are inherent in their system of illumination and depend on the wave length of light. The shorter the wave length of the radiations, the

\* One Ångström Unit ( $1\ \text{Å}$ ) equals  $1 \times 10^{-8}\ \text{cm.}$  or one ten-thousandth of a micron ( $0.0001\ \mu$ ).



object, higher resolution is obtained, and the image formed, invisible by direct illumination, can be caught by the camera's eye. Thus by ultraviolet microscopy the limit of resolution is increased to about  $0.1 \mu$  and the maximum useful magnification to about 2000 diameters.

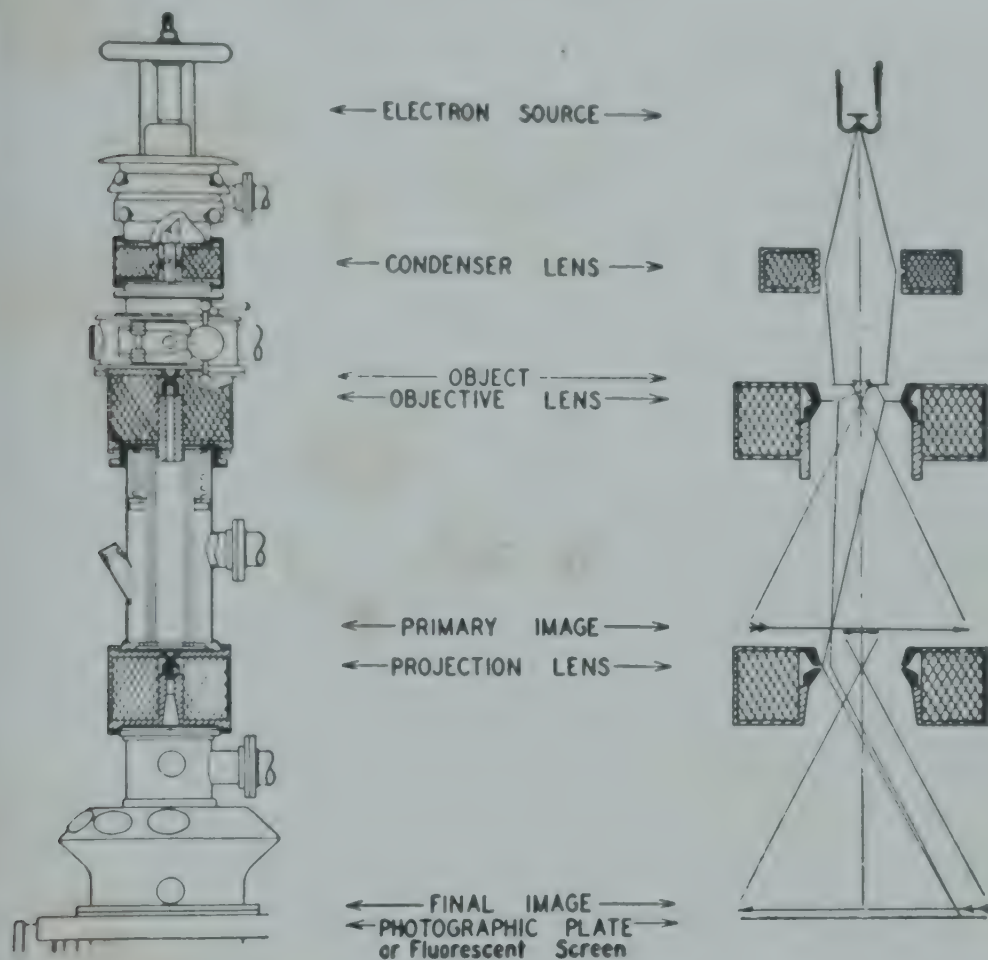


Fig. 94. The "optics" of the RCA electron microscope. On the left is a scale drawing of the instrument and on the right a schematic diagram of the instrument showing the arrangement of lenses and the formation of images. (From T. F. Anderson in *Advances in Colloid Science*, vol. I, Interscience Publishers, Inc., New York, 1942, p. 356.)

**Electron Microscope.** The electron microscope surmounts the limitations of light microscopy by utilizing the extremely short waves associated with high-speed electrons. A 50,000-volt electron corresponds to a wave length of  $0.0535 \text{ \AA}$ . With this instrument then a resolving power of about  $10 \text{ \AA}$  and a useful magnification one hundred times greater than that of the ultraviolet microscope may be obtained.

The "optics" of the electron microscope (Fig. 94) and of the compound light microscope may be compared. In the latter a beam of light travels from its source, usually an incandescent lamp, through air and a series of lenses. Entering the object mounted on a glass slide the light is modified (by a number of actions including absorption, refraction and scattering) to form an image which is magnified by the lenses of the objectives and the eyepiece. In the

electron microscope a heated filament serves as the source of electrons. The electrons are accelerated according to the voltage applied, and travel at a high speed through a vacuum. Thus electron beams are substituted for light beams. Magnetic fields replace the lenses of the light microscope. When the electron beams encounter the object they are scattered to different degrees depending on the density and thickness of the object's structures. Only the thinnest or least dense portions of an object such as a bacterium will allow the passage of electrons. The image formed and magnified by the magnetic lenses is then a shadow of the object revealing the different opacities of its parts to electrons.

To produce satisfactory images specimens must be thin (not over about 1  $\mu$  thick), and must be dried either before or after mounting on a suitable support. Bacteria, viruses and other particles are dried on a thin film of collodion or similar plastic material which is supported by a fine wire mesh mounted in the specimen holder. The mount is inserted into the specimen chamber of the microscope, the system is evacuated, and the voltage applied. The operator manipulates various control knobs on the instrument's panel to move the preparation to different fields, focus and regulate the brightness of the image. Magnification can be varied from 800 to 25,000 diameters. The image of the object as it appears on a fluorescent screen is observed through a viewing chamber. A permanent record of the image is made by closing the viewing chamber and exposing a photographic plate. Since photographic enlargement allows further magnification, electron micrographs commonly show bacteria, rickettsiae and viruses magnified 20,000 to 100,000 times.

Recently a shadow-casting technique has been developed which produces a three-dimensional effect in photomicrographs made with the electron microscope. One side of the particles, such as viruses, are coated with a thin film of a metal, usually gold, and in the resulting micrograph a shadow appears on the uncoated side, thus bringing out the depth and shape of the particle.

## PREPARATION OF MICROSCOPIC MOUNTS

**The Simple Liquid Mount.** A drop of liquid containing the microorganisms is placed on a clean plane glass slide and covered with a clean coverslip. This type of mount is usually examined under the low or high dry objectives, and is commonly employed in the study of the larger microorganisms such as the protozoa, molds, yeasts and the unicellular algae. It is also used to study spirochetes under the darkfield microscope. The organisms are alive and generally unstained when observed in this preparation, but vital stains may be applied. A variation of the ordinary liquid mount may be used to prevent crushing the delicate structures of molds. A small piece of a mold colony plus the agar in which it is growing is removed intact from a plate culture and placed, mold uppermost, on a slide where it is moistened with a few drops of alcohol run in from the sides of the specimen. In the same way enough glycerol (glycerin) is



ed to form a layer thick and high enough to prevent the weight of the cover- from distorting the aerial portion of the plant. Similarly, mold may be mounted in a single liquid, lactophenol, which is a solution of phenol, lactic acid glycerol (see Appendix 2).

**The Hanging Drop.** This preparation is suitable for studying motility of bacteria. A thin rim of vaseline is applied just outside the edge of the depression in a clean, hollow ground, glass slide. A small high drop of the bacterial culture is placed exactly in the center of a clean coverslip. The slide is then inverted over the coverslip so that the drop is just in the center below the hollow. The pressure spreads the vaseline and sticks the coverslip to the slide, creating a sealed chamber. When the entire preparation is inverted the drop hangs down into this chamber. The advantages of the hanging drop over a simple liquid mount include the absence of pressure on the drop in the former and its ability to prevent rapid evaporation. The drop is located by the low dry objective, but only

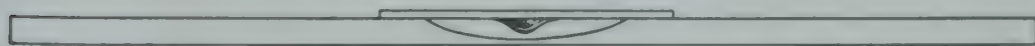


Fig. 95. Hanging drop preparation.

h a higher powered objective and partially diminished light can most unstained bacteria be seen. All small particles, animate and inanimate, motile and nonmotile, exhibit **Brownian movement**, a jiggling in place due to the bombardment of each particle by molecules in the surrounding liquid. True motility of bacteria is evidenced by translocation of a cell from one place on the slide to another.

**The Fixed Stained Mount.** In this mount a drop of the material to be examined is spread out in a thin film over a grease-free plane slide and allowed to dry thoroughly so that particles, including bacteria, become stuck or **fixed** to the slide. If the specimen is a liquid, for example a broth culture, a sterile looped inoculating needle is used to remove a drop which is then smeared out over the slide. In the same way specimens from laboratory animals or patients, sputum, spinal fluid, throat swabs, pus or various other exudates can be spread out with a wire loop or a sterile swab to make a direct microscopic examination of these materials. If a smear is to be made from a colony or a slant culture of bacteria, a small drop of water is first placed on the slide and a minute amount of the bacterial growth is emulsified in the drop before spreading it out. How thick the smear should be depends chiefly on the density of the material. A satisfactory film dries quickly after it is spread out and is barely cloudy after drying. When the film appears to be perfectly dry it is fixed by passing the slide rapidly through a flame three times to remove the last trace of moisture. To be sure the slide does not become overheated it is a good idea to pass the fingers through the flame with it. The fixed film is then stained by one of the several methods described below and is examined under the oil immersion objective. The combination of high magnification and staining reveals structures of the bacterial

cell which can be seen with difficulty or not at all in liquid mounts. On the other hand the microorganisms in the fixed film are no longer alive.

**Permanent Mounts.** Fixed stained films can be used repeatedly and indefinitely or until the stain fades if, after examining, the film of oil is covered with xylene and this solvent is removed by blotting with filter paper or by drawing a piece of lens paper through the drop. Films may be protected by covering them with a thin coverslip. In this type of permanent mount a drop of Canada balsam is placed on the smear and the coverslip is pressed down gently over it to form a thin layer between the two glass surfaces. When the balsam is firm and dry the slide may be examined as usual except that the drop of immersion oil is placed on the coverslip, from which it can be wiped after each examination.

### STAINING METHODS

**Simple Staining.** When a single dye is applied to a fixed film of bacteria or other microorganisms the method is known as simple staining. The most widely used simple stain is Löffler's alkaline methylene blue, but others, including gentian violet, basic fuchsin and safranin, are also satisfactory in routine bacteriological work. Methylene blue is poured over the fixed smear and allowed to act for one minute. Then the slide is washed thoroughly in water until no more color leaves the smear. It is dried in the air or by blotting (not wiping) with absorbent paper.

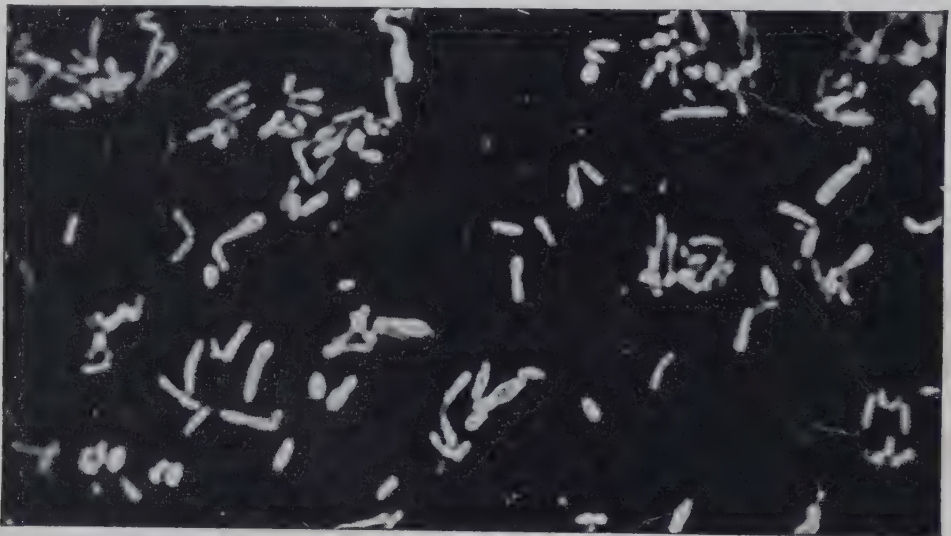


Fig. 96. Photomicrograph of diphtheria bacilli (*Corynebacterium diphtheriae*) as revealed by negative staining with Congo red. (From Henrici: *The Biology of Bacteria*, D. C. Heath & Co.)

**Negative or Relief Staining.** Bacterial stains color bacteria more deeply than anything else on the slide and if the smear is properly made from a bacterial culture the background is clear and colorless. If, however, a loopful of a suspension of bacteria is mixed with a loopful of a dark, finely particulate



substance such as nigrosin or India ink and the mixture is spread out over the slide in a thin film and dried, the bacteria appear colorless or white against a dark background.

**The Gram Stain.** In 1884 Gram developed the differential staining method which now bears his name. This method divides all bacteria into two categories: the gram-positive and the gram-negative forms. The bacteria are stained first with carbol gentian violet (or crystal violet) and next with Gram's iodine solution. At this point all bacteria are colored dark purple. The slide is then covered with 95 per cent alcohol for a few seconds. The alcohol decolorization is the decisive step in the procedure, in separating the gram-positive cells which retain the purple color from the gram-negative cells which lose the stain and again become colorless. If the alcohol is allowed to remain on the slide for too long a time, gram-positive bacteria may also be decolorized. Moreover, gram-positive bacteria often become gram-negative in old cultures.

Since gram-negative bacteria are colorless after treatment with alcohol and are therefore difficult to see, a counterstain is selected which tints these cells but does not obscure the initial color of gram-positive forms. Safranin or dilute basic fuchsin are popular counterstains and consequently gram-negative bacteria are generally stained pink. There is good evidence that the two reactions are due to fundamental differences in the chemical composition of the surface of the two types of cells, and that the substance responsible is magnesium ribonucleate. This substance is present in the outer layer of gram-positive cells and is lacking in gram-negative cells. When gram-positive bacteria are stripped of their outer layer (experimentally or by aging), the underlying gram-negative core of the cell is exposed.

Directions for the Gram stain may be summarized briefly as follows:

1. Flood dried, fixed film with carbol gentian violet for  $\frac{1}{2}$  minute. Wash thoroughly. Shake off excess water.
2. Cover with Gram's iodine solution for 1 minute. Wash.
3. Decolorize with 95 per cent alcohol (or equal parts of acetone and 95 per cent alcohol) for about 10 to 15 seconds or until no more dye leaves the smear. **Wash immediately.**
4. Counterstain with safranin or dilute fuchsin for 30 seconds. Wash.
5. Blot off excess water. Dry in air. Examine with the oil immersion objective.

**Acid-Fast Stains.** It is difficult for stains to penetrate cells of the mycobacteria because of their high lipoid content. Only after prolonged treatment with carbol fuchsin, one of the most penetrating stains, or the application of heat during the staining process, can these bacteria be colored. Once stained, however, the waxy material of the cells holds the carbol fuchsin even in the presence of acid alcohol. The steps of the **Ziehl-Neelsen** method which demonstrate the property of acid-fastness are:

1. Smear out material (cultures or specimens), dry, and fix as usual.
2. Cover smear with carbol fuchsin and steam over a low flame or a water bath for 5 minutes. The stain should never boil nor should it be allowed to dry.

3. Let slide cool a minute. Wash thoroughly in water.
4. Decolorize in acid alcohol (95 ml. of 95 per cent ethyl alcohol plus 5 ml. concentrated HCl) for 5 minutes or until no more color leaves the smear.
5. Wash in water.
6. Counterstain with methylene blue for 30 seconds.
7. Wash, dry and examine with oil immersion objective.

By this method acid-fast bacteria appear red against a blue background.

For other methods which test for acid-fastness see the Appendix.

**Demonstration of Capsules.** A combination of negative and simple staining provides a satisfactory means of demonstrating the presence of capsules. Certain bacteria, such as *Klebsiella pneumoniae*, develop large capsules when grown on infusion agar enriched with blood or serum. A very small amount of moist growth is removed from the bottom of a slant culture and mixed with a loopful of 6 per cent dextrose solution on a clean slide without spreading the drop. A loopful of nigrosin solution or India ink (previously examined to be sure it is relatively free from bacteria) is then added and blended with the cell suspension as the mixture is spread out evenly in a thin film. The slide is next thoroughly dried in the air, after which it is stained with methylene blue or safranin for 1 or 2 minutes. This stain is washed off carefully with water and the smear is dried without blotting. Under the oil immersion lens the capsules are seen as clear halos around the blue or red colored cells and surrounded by the dark gray or black background.

**Demonstration of Flagella.** Bacterial flagella are such slender strands of poor staining substance that they are not visible in ordinary microscopic preparations. Their demonstration calls for darkfield illumination or special methods, such as the silver impregnation and flagella stains, which include mordants to coat these delicate filaments in order to increase their size and to fix the stain to their surfaces. Flagellar staining techniques are often difficult to master, but a relatively simple and usually satisfactory method has been introduced by Leifson. The following recommendations are, in the main, taken from Leifson's directions for the preparation and use of his flagellar stain:

According to this method the bacteria are removed from the surface of a moist agar slant culture and suspended in distilled water. The slightly cloudy suspension is incubated for a few minutes after which a drop is transferred to a clean slide. The condition of the slide used in making the preparation largely determines the success of the method. New slides handled individually with forceps should be soaked in cleaning solution for 1 or 2 days, and then washed thoroughly, first in tap and, finally, in distilled water. They should not be wiped, but should be drained and air dried standing upright. When dry they must be protected from dust and lint. Before making a smear, the slide is flamed on one side until the flame turns orange colored. When the slide is cool, a large loopful of the bacterial suspension is placed on the previously flamed side about  $\frac{3}{4}$  inch from one end of the slide which is then elevated to allow the liquid to run down over most of its surface. The slide should remain in this position until the smear is dry and it should not be fixed by heat.



**Leifson's Flagella Stain.**

1. Prepare stain and mordant as directed for powdered stain or by mixing the following ingredients:

Tannic acid	0.85 gm.
Sodium chloride	0.5 gm.
Para-rosanilin acetate	0.35 gm.

Dissolve the above substances in a mixture of:

Alcohol (95 per cent)	35 ml.
Distilled water	65 ml.

Let stand about 10 minutes, shaking several times to dissolve. The stain is ready to use when the ingredients are completely dissolved.

2. Flood the entire smear with a generous amount of the stain until it is "heaped up" on the slide and allow it to act for from 8 to 15 minutes at room temperature or until a metallic scum forms over the surface.
3. Wash thoroughly in tap water.
4. A counterstain of borax methylene blue (0.1 gm. methylene blue and 0.2 gm. borax in 100 ml. distilled water) may be applied for 10 minutes after which the slide is washed in tap water. This step may be omitted.
5. Drain, do not blot, dry, and examine with the oil immersion objective.

**Spore Stain.** Since bacterial spores are refractile to ordinary staining, special methods have been devised to color them. Once stained, the spores are not as easily decolorized as the vegetative cells. This is the principle involved in the following method.

1. Emulsify some of the growth of a sporulating bacillus from a 2- to 3-day-old agar slant in a drop of water and smear out on the slide. Dry and fix as usual.
2. Cover the slide with concentrated carbol fuchsin and steam for 3 to 5 minutes over a low flame or a water bath, never heating enough to boil the dye. Do not allow it to dry.
3. Cool slide a minute before washing thoroughly in water.
4. Cover the smear with a decolorizing agent. Recommended are:
  - 95 per cent alcohol for 20 to 30 seconds,
  - 5 per cent acetic acid for 2 seconds,
  - 1 per cent hydrochloric acid for 2 to 3 seconds,
 or repeated applications of acetone over a period of 5 seconds.
5. Wash immediately in water.
6. Counterstain with methylene blue.
7. Wash, dry and examine with the oil immersion objective.

**MICROMETRY**

Bacteria and other microscopic bodies are measured in terms of microns. A micron (represented by the Greek letter  $\mu$ ,  $\mu$ ) equals 1/1000 of a millimeter or 25,000 of an inch. When measuring microorganisms, for example bacteria, as observed through an **ocular micrometer**, a scale which is inserted between the lenses of the eyepiece of the microscope. The length and width or diameter of the cell is measured by the eyepiece scale in terms of ocular

micrometer units, but these are not the actual dimensions. They are merely the number of ocular units occupied by the length and width of the magnified cell. To estimate its actual size a special slide known as a **slide micrometer** which has been engraved with lines one-tenth of a micron ( $0.1 \mu$ ) apart must replace

the slide bearing the bacteria. By comparing the scale on the ocular micrometer with the micron "yardstick" on the slide, one can express in microns the size of each ocular unit. Knowing the micron value for the unit the dimensions of the bacterial cell can be stated in terms of microns (Fig. 97).

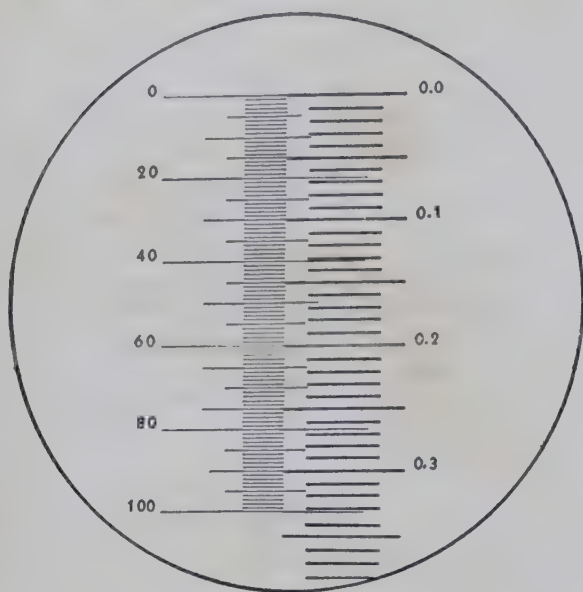


Fig. 97. Calibration of ocular micrometer disc. The scale to the left, marked 0 to 100, is that engraved on the ocular micrometer disc. Seen through the ocular micrometer disc is the scale of the slide micrometer (right) on which each division equals  $0.01$  mm. (Courtesy of American Optical Co., Scientific Instrument Division.)

### MICROSCOPIC COUNT OF BACTERIA

The number of bacteria in a given unit of liquid may be counted by direct microscopic examination. If a known amount of material is spread over a known area on a slide and examined with the microscope so adjusted that the exact size of the visible field is known, the number of organisms contained in the sample may be computed. This method de-

termines the total number of cells but does not distinguish between living and dead cells. The microscopic count is used in determining the number of bacteria in a sample of milk and in a vaccine. Directions for the Breed method for the direct microscopic count of bacteria in milk are given in the Appendix.



# 10

## LABORATORY EQUIPMENT AND STERILIZATION METHODS

Much of the glassware and other apparatus used in microbiology will be familiar to the beginning student from previous experience in chemistry and biology. While there are some instruments especially designed for the study of microscopic organisms, the outstanding differences between the equipment of the microbiology laboratory and other laboratories depend on the necessity for asepsis in microbiological techniques. **Asepsis** means essentially freedom from contamination. Aseptic techniques have two general objectives: to prevent the entrance of undesirable and foreign microorganisms and to prevent the escape of microorganisms. For example, all organisms from the surroundings (*i.e.*, the air, dust, tables or the bacteriologist's body) must be excluded from laboratory cultures. On the other hand, the organisms in the laboratory cultures must not be allowed to escape into the surroundings. In short, the purpose of aseptic technique is to control microbic traffic.

To determine the kinds of microorganisms in a given specimen, all extraneous organisms must be excluded, and in order to identify many microorganisms they must be grown alone as a **pure culture**. It is obvious, then, that culture media, glassware and instruments used in manipulating and growing microorganisms must be free from all life at the start, *i.e.*, they must be **sterile**. Moreover, they must be constructed and handled in such a way that they remain free from contamination.

### LABORATORY APPARATUS

In addition to the usual laboratory supplies of slides, test tubes, flasks, beakers, burettes, Bunsen burners and the like, the microbiologist uses glassware and equipment particularly suited to working with invisible, living organisms.

**Inoculating Needles.** An inoculating needle is a piece of platinum or some alloy wire fused into the end of a glass rod or fitted into a metal handle. The length of the wire is generally about four inches, but may be adjusted to suit the worker. The free end of the wire is bent to form a loop to hold a drop of liquid or it is left straight. The wire loop and straight needle are used in introducing microorganisms into new media, *i.e.*, inoculating (L., *inoculare*, to ingraft) test tubes and plates of nutrient substances, or to remove samples from specimens or

cultures. Thus, the wire loop is used to pick up a drop of fluid containing microorganisms and to spread it out on a glass slide, while a straight needle is used to "pick" a bit of a bacterial colony, to emulsify it in a drop of water for microscopic study or to transfer the growth to other nutrient media.

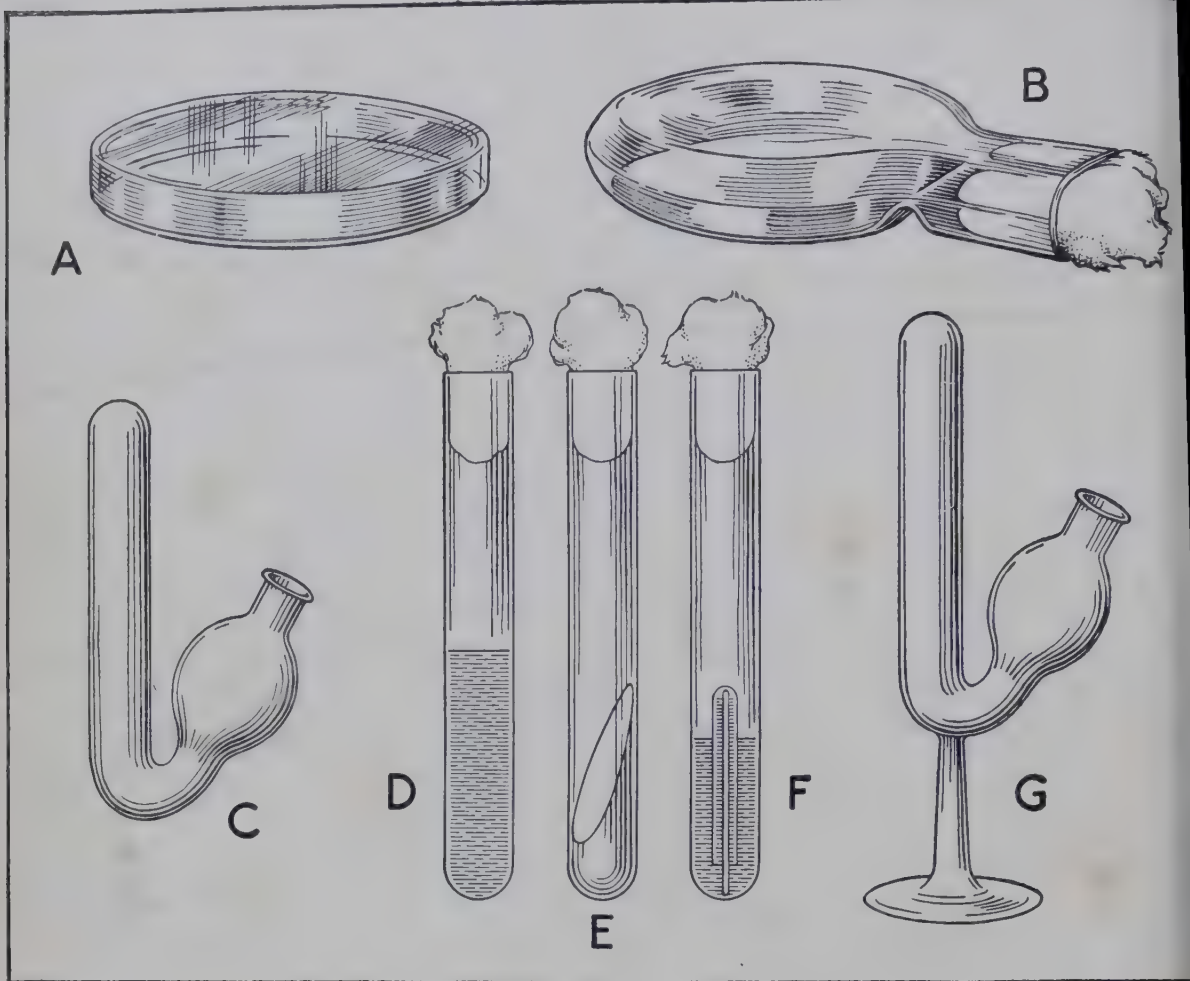


Fig. 98. Glassware used in the cultivation of bacteria. A, Petri dish; B, Kolle flask; C, Smith fermentation tube without stand; D, test tube with liquid medium; E, test tube with agar slant; F, Durham fermentation tube containing an inverted vial to act as a gas trap; G, Smith fermentation tube with stand. (From Belding and Marston: *A Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)

**Petri Dishes.** Richard J. Petri, an assistant in Koch's laboratory, introduced a round, covered, glass dish suitable for growing microorganisms in a liquefiable solid medium. This dish was a decided improvement over Koch's original method of pouring gelatin or agar on sterile glass slides and covering them with a bell jar, for its top was deep enough to cover completely the inside of the dish and to protect it from aerial contamination. Petri plates universally used in bacteriological laboratories today are made in several sizes, but the usual ones are 10 cm. in diameter.

**Fermentation Tubes.** In studying the fermentative action of microorganisms it is important to know whether or not gas is produced. For this purpose



ial tubes have been devised to catch gases which would escape from ordinary tube cultures. One kind, the **Smith fermentation tube**, has its closed end bent up into an arm which acts as a trap for gas. Another device for catching gas is used in the **Durham fermentation tube**, an ordinary test tube in which a small, hollow vial is inverted in a carbohydrate broth. The vial must be completely filled with the liquid medium before inoculation. If the organisms growing in the medium ferment the carbohydrate with the formation of gas, some of the liquid in the vial will be displaced by this gas.

**Incubators.** Laboratory incubators are insulated chambers equipped with thermostat controls which maintain a constant temperature. They serve as a means to store cultures at the temperature required to foster the growth of the microorganisms in those cultures. Many bacteria grow best at a temperature near that of the human body and, therefore, incubators are often set at  $37^{\circ}\text{C}$ . Some organisms develop best at room temperatures ( $20^{\circ}$ – $24^{\circ}\text{C}$ ) and others at  $40^{\circ}\text{C}$ , and incubator temperatures are regulated accordingly.

### CLEANING GLASSWARE

Glassware must be chemically clean and dry before use. Dirty glassware contaminated by infectious microorganisms must be disinfected, *i.e.*, the disease-producing microbes must be destroyed by appropriate chemicals, boiling or autoclaving. Then it must be cleansed thoroughly, usually by cleaning solution or detergents, such as tri-sodium phosphate, followed by rinsing first in running tap water and finally in distilled water. Immediately after working with infectious materials, pipettes should be placed tips down in a cylinder of enough 1 per cent lysol or similar disinfectant to cover the contaminated surfaces and left in it long enough to kill the infectious organisms before washing.

New glassware should be soaked for several hours in potassium dichromate-sulfuric acid cleaning solution, in 2 per cent hydrochloric acid or in a detergent solution, then washed and rinsed before being used. Cloudiness may be removed from glassware by soaking in cleaning solution for 24 hours followed by repeated rinsings. As described under methods of sterilization, glassware must be protected from aerial contamination by cotton plugs, wrappings or containers before sterilizing.

### METHODS OF STERILIZATION

**Sterilization** is the process of freeing an object or material from all living microorganisms. A sterile object is free from harmless saprophytes as well as dangerous agents of disease. Sterilization is usually accomplished by exposure to dry heat for sufficient time to kill all kinds of microorganisms. The intensity of heat and the time necessary for complete destruction of all life will naturally depend on the resistance of the contaminating organisms. Since microorganisms vary in their resistance to heat and chemical agents it is essential that sterilization methods be adequate to kill the most resistant organisms. Certain bacteria form

spores which are the most resistant forms of life known, for they may be boiled for minutes or even hours and still remain alive. Since these hardiest of microorganisms are among the most common in our surroundings, it is clear that ordinary boiling will not guarantee sterilization in the true sense of the word. Although the boiling of instruments for a few minutes is sufficient to kill non-sporulating bacteria, it does not destroy the toughest bacterial spores. Fortunately, since most disease-producing bacteria do not form spores, boiling is a safe method

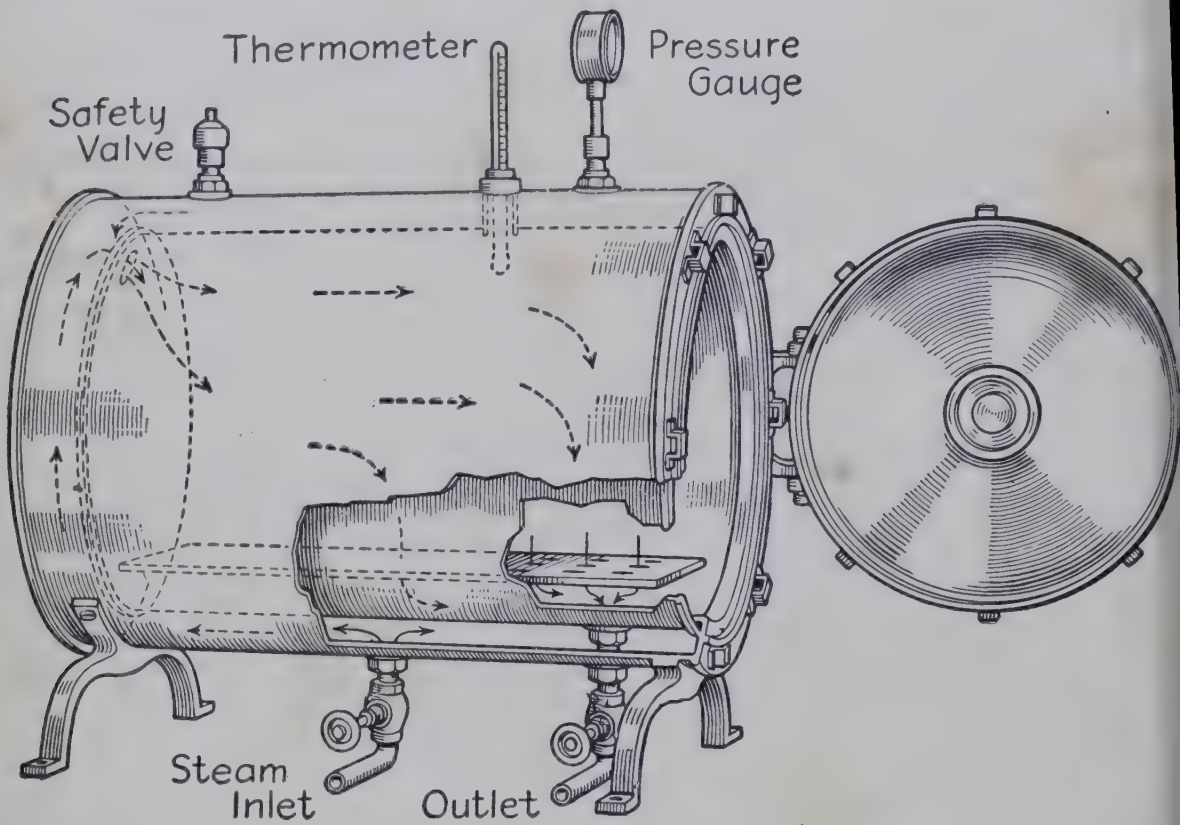


Fig. 99. Autoclave. (From Belding and Marston: *A Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)

for many procedures. However, if true sterility is required, as it is in bacteriological work and in surgical procedures, more rigorous treatment is necessary.

**Sterilization by Steam Under Pressure. The Autoclave.** Flowing steam under pressure is the most effective method of sterilization. This is accomplished in the autoclave, an apparatus which compresses steam in a closed, air-free chamber, measures the steam pressure and can be regulated to the degree of heat required. The more pressure applied to the steam the higher its temperature is raised above  $100^{\circ}\text{C}$ . The temperature of steam subjected to 15 lbs. pressure at sea level is approximately  $121^{\circ}\text{C}$  ( $250^{\circ}\text{F}$ ), and the time required to sterilize at this temperature depends on the volume and the nature of the objects to be sterilized. Large flasks of solutions require a longer time than test tubes of the same fluid. Heat penetrates through material by convection currents; hence liquids are sterilized more readily than solid materials.



Sterilization is accomplished usually at  $121^{\circ}\text{C}$  (15 lbs. steam pressure) for 15 to 30 minutes. The autoclave is used to sterilize organic matter which would be ruined by baking in the high temperatures of dry ovens or by burning in an open flame, and also to sterilize all other materials which can be protected from or are not injured by the moisture of the steam. The *Arnold dressing sterilizer* is an autoclave equipped with a vacuum mechanism

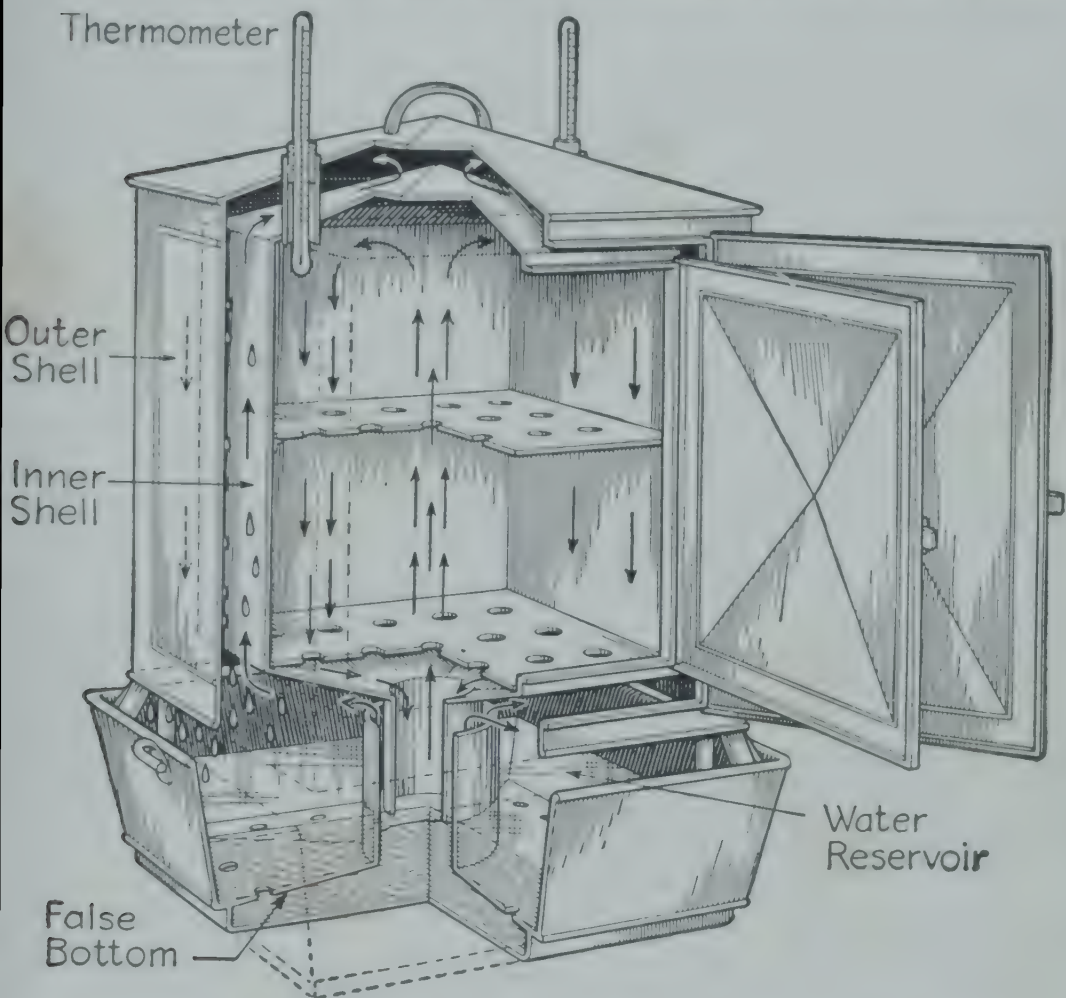


Fig. 100. Arnold steam sterilizer. (From Belding and Marston: *A Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)

removing the steam after the sterilizing process, thus eliminating the moisture condensation which collects on cooling objects in the ordinary autoclave. In the laboratory, culture media and solutions are sterilized by autoclaving. Materials to be prepared for autoclaving in such a way that they remain free from contamination when they are removed from the sterilizing chamber. Tubes and syringes are plugged with nonabsorbent cotton; baskets of tubes and the mouths of flasks may be further protected by paper covers. Sheets, towels, gowns, dressings and the like are arranged in packs wrapped in muslin cloth which are then placed in metal containers or drums.

**Intermittent Sterilization. The Arnold Sterilizer.** John Tyndall (1877) discovered that whereas a single one hour exposure to boiling temperature

might not kill every microbe in a nutrient infusion, boiling for one minute five successive occasions with intervals of several hours at room temperature would succeed in sterilizing. We now know that the explanation of this intermittent sterilization lies in the life cycle of spore-forming bacteria. Bacterial spores which are not killed by one exposure to heat are permitted to germinate

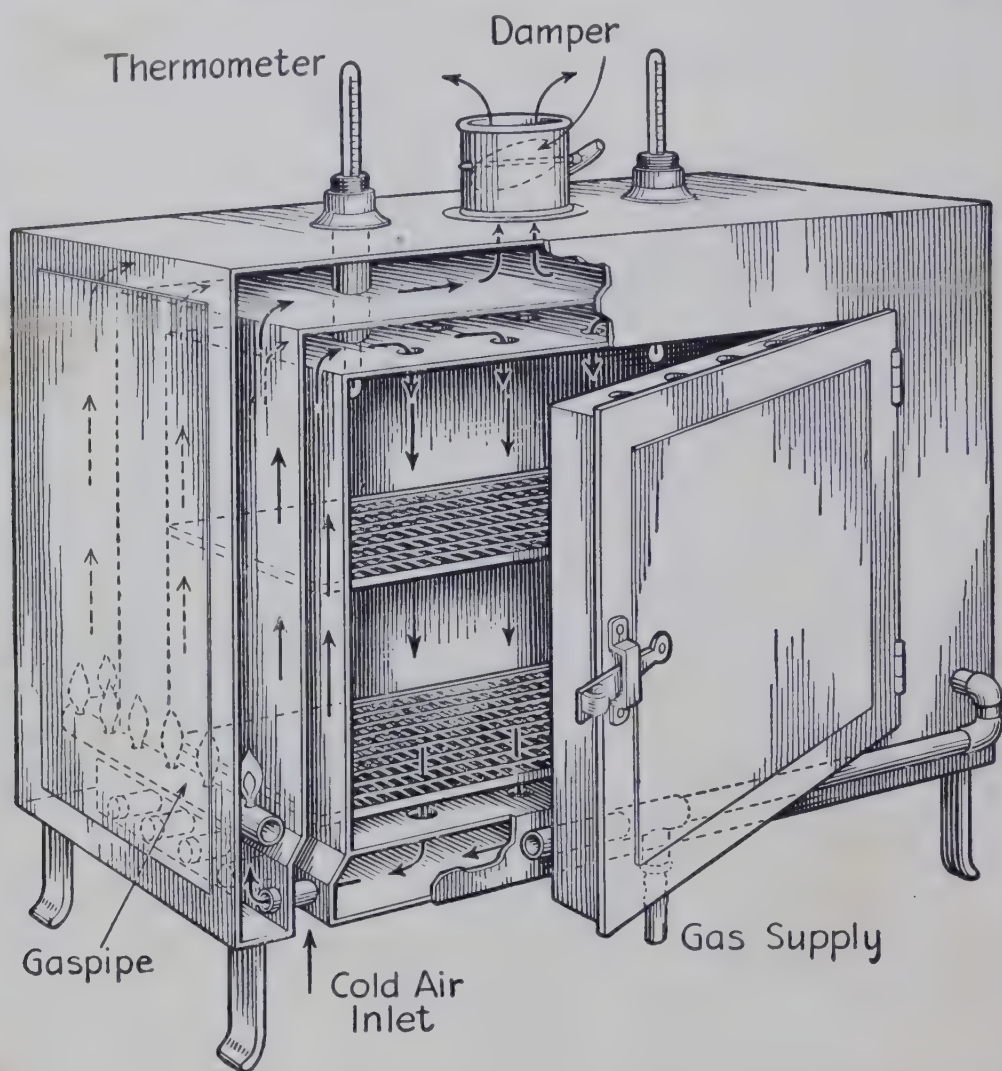


Fig. 101. Hot-air sterilizer. (From Belding and Marston: *A Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)

into their nonresistant vegetative form during the interval when they are held at room temperature. The vegetative cells are then destroyed by the next heat treatment. A more efficient method is that of circulating live steam ( $100^{\circ}\text{C}$ ) in an Arnold sterilizer for one hour on each of three successive days. The material to be sterilized is held at room temperature during the interims to permit germination of surviving spores. Intermittent sterilization is used today only for materials that would be harmed by higher temperatures.

**Sterilization by Dry Heat.** In the above descriptions of sterilization methods moist heat has been the agent employed. It is more practical to use dry heat in the sterilization of some articles, but since dry heat is less effective



moist heat in killing microorganisms, the temperature must be higher and the time of exposure more prolonged than in the case of moist heat sterilization. Materials unharmed by such temperature can be sterilized by baking in hot ovens at  $160^{\circ}\text{C}$  ( $320^{\circ}\text{F}$ ) for two or more hours. The hot air method is routinely used in sterilizing Petri plates, pipettes, flasks and test tubes. Cottons, scalpels, blades, surgical needles, syringes and syringe needles may also be sterilized by dry heat if over-heating is avoided.

Before sterilization, steps must be taken to protect these articles from contamination after removal from the oven. Empty tubes and flasks are stoppered with cotton plugs and capped with paper. Pipettes, plugged at the mouth end by a piece of absorbent cotton, and Petri plates are placed in metal boxes or wrapped in paper. Syringes are wrapped in cloth or enclosed in glass tubes or cylinders. The needles are sewed through gauze and protected with the folds of a gauze bandage, while syringe needles and scalpels are held in glass vials or tubes. Cotton and paper will char if the temperature rises above  $180^{\circ}\text{C}$  for any length of time. Metals and glassware should be heated and cooled gradually.

**Incineration.** Burning sterilizes since it completely destroys all living organisms, but the object to be sterilized may also be destroyed by the process. The metals withstand heating to such an extent that they may be held in a furnace until red hot and still remain intact. Of this nature is the platinum or nichrome wire used in inoculating needles, and the bacteriologist sterilizes his hands by flaming until the entire length of the wire glows red. In some procedures the tips of forceps or other metal and glass objects may be sterilized over a flame, but such treatment is not ideal; proper plans should include the sterilization of these articles by autoclaving or baking.

**Sterilization by Filtration.** This method is different from the ones previously described, for whereas heat sterilizes by killing microorganisms, filtration sterilizes by removing microorganisms. Heat-sterilized materials are free from living microorganisms, but the dead cells have not been removed. Bacteria suspended in fluids are held back by fine-pored filters made of compressed porous materials. There are several varieties differing as to composition and construction of the filter itself and the structures which support the liquid to be filtered, force it through the filter, and collect the filtrate.

Chamberland filters are made of unglazed porcelain and are shaped like hollow candles with one open and one closed end. The liquid enters the open end of the filter and passes through it into a sterile collecting flask. Berkefeld filters are hollow candles of diatomaceous earth which stand closed end upright in a surrounding glass mantle which is designed to hold the fluid to be filtered. Both these candle filters are manufactured in different grades according to pore size. There are Berkefeld V (German, *viel*) or coarse, N (*normal*) or medium and W (German, *wenig*) or fine filters, while grades of Chamberland filters are designated L<sub>1</sub>, L<sub>2</sub>, L<sub>3</sub>, etc. Filter candles must be cleaned thoroughly after each filtration and discarded when the pores become clogged.

Another entirely different type is the Seitz filter which consists of an asbestos pad fixed firmly in place at the bottom of a metal cylinder which holds the liquid to be filtered. A new Seitz pad must be used for each filtration. Sintered (frit) glass filters are becoming increasingly popular in bacteriological work since they can readily be made chemically clean and are adaptable for filtering large amounts of material. These filters are made by heating finely ground, Pyrex glass

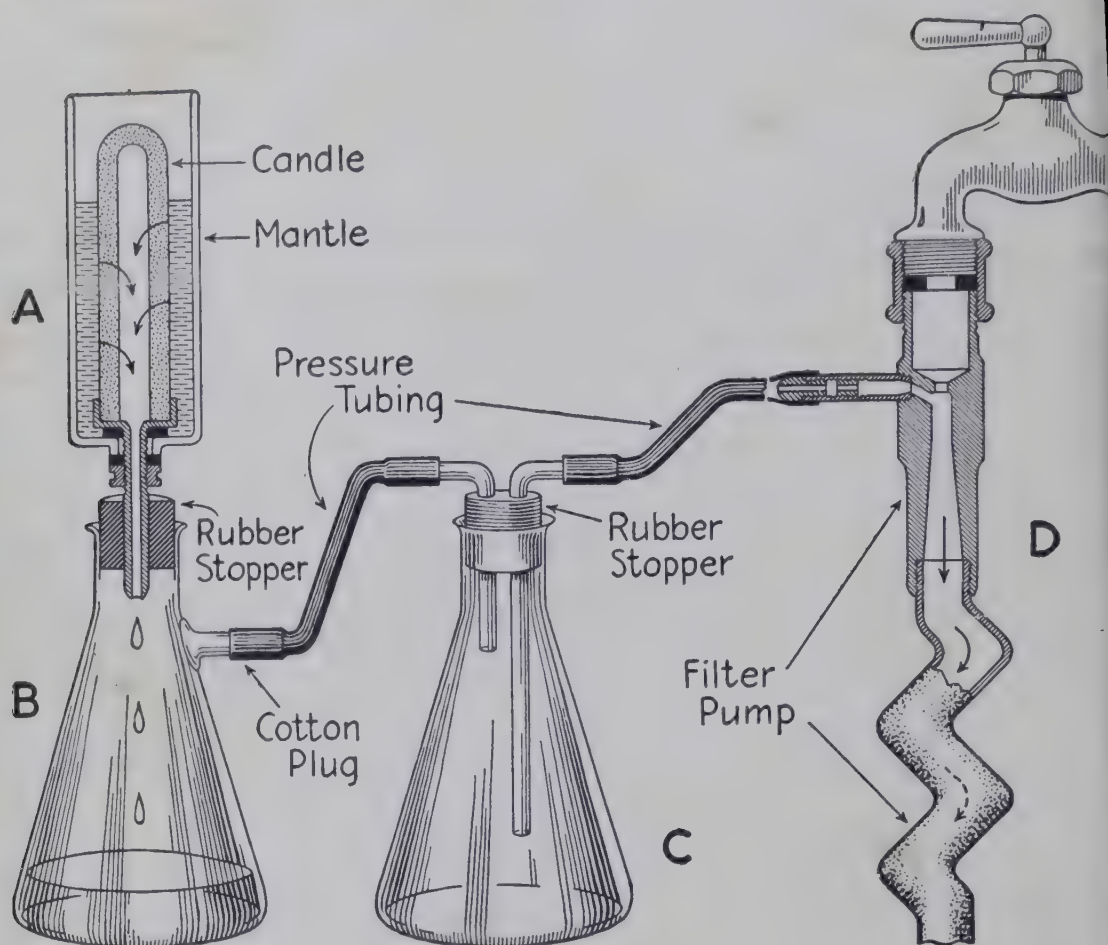


Fig. 102. Typical filtration apparatus. A, Berkefeld filter; B, collecting flask containing filtrate; C, trap; D, suction pump. (From Belding and Marston: *A Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)

particles of uniform size in a disc-shaped mold, and fitting the resulting porous glass filtering disc into a funnel-shaped receptacle.

Fluids are forced through all these filters by pressure, either as positive pressure on the liquid above the filter or as negative pressure created by suction in the collecting flask below the filter. The completely assembled filtration apparatus is sterilized by autoclaving before each operation. Filtration is used for the sterilization of sugar solutions and other fluids, such as serum, which should not be subjected to high temperatures. It is also used to prepare sterile solutions of microbial products, such as enzymes and exotoxins, by separating them from the microorganisms which produced them, and to separate the filtrable virus from bacteria and other microorganisms.



## CULTIVATION OF MICROORGANISMS

## CULTURE MEDIA

More can be learned about microorganisms by growing them than by observing their appearance under the microscope. In microbiology the term culture is used to denote a population of microorganisms in an environment (or medium) which supports their growth, while the nutrient substances in which the organisms grow is the **culture medium**.

**Liquid and Solid Culture Media.** The first culture media were liquids. Leeuwenhoek first grew bacteria and protozoa in stagnant natural waters and infusions. Decoctions of hay and other vegetables, milk and even urine were used by early bacteriologists. Pasteur worked with transparent liquid media. It was not until Koch grew bacteria on pieces of raw potato, a method borrowed from the mycologists, that the value of solid media was recognized. In 1881 he produced a liquefiable solid culture medium made of clear nutrient fluid mixed with gelatin which he used in tubes or poured out on the flat surfaces of sterile glass slides and plates.

Agar, a gelatinous substance extracted from Japanese seaweed, was then brought to Koch's attention presumably by Frau Hesse, a worker in his laboratory. Koch soon realized that agar had important advantages over gelatin as a solidifying agent, since it remains solid at warm room and body temperatures which are optimum for many microorganisms. Gelatin is liquefied even at cool temperatures by the digestive action of many organisms, but agar-digesting organisms are rare. Nutrient gelatin and nutrient agar are today standard culture media. The media may be poured when warm, and liquid into any suitable container, such as ordinary glass tubes, Petri plates or larger vessels, including beakers and flasks. Not all solid media are liquefiable, nor do they necessarily contain gelatin or agar. For example, the heat-coagulated proteins of egg or blood do not solidify the medium permanently. If blood, serum or ascitic fluid is added to nutrient agar it is no longer liquefiable.

**Requirements for a Satisfactory Culture Medium.** Microorganisms, like all other plants and animals, require moisture, suitable food, a certain amount of mineral salts, and the right concentration of hydrogen ions in their surroundings. The medium must contain no injurious chemicals which inhibit growth or kill the original cells and, for satisfactory laboratory studies,

it must be **sterile** at the start. Other environmental conditions must be regulated when organisms are introduced into the culture medium, for they differ in their optimum temperature and oxygen requirements. Most fungi grow best in the dark or away from bright light.

**Moisture.** Microorganisms must have an adequate supply of water for all exchanges between the cells and their surroundings and the metabolic chemical changes within the cells must take place in a fluid medium. Thus, foods and oxygen enter the cells and wastes leave the cells only when they are dissolved in water. No living cells can grow when they are completely dry although they can survive in this state for some time under artificial conditions.

**Food.** Growth is a process of increase of protoplasm through the conversion of foodstuffs into the body substance. The problem of finding suitable food for a particular organism then is one of discovering the substances which the organism can convert into the constituents of its protoplasm and which provide an available source of energy. The green plants can, by virtue of their chlorophyll, use light energy to synthesize the complex organic compounds of the protoplasm from inorganic substances, namely water, carbon dioxide, nitrates and other salts. Protozoa and fungi, lacking the green coloring matter chlorophyll, are devoid of this photosynthetic power. Although certain of the microscopic fungi can grow on simpler substances, most culture media supply the yeasts, molds and bacteria with the complex organic compounds of meat extracts, meat infusions and partially digested proteins such as peptones.

**Osmotic Pressure.** When a living cell is bathed in distilled water or a hypotonic solution containing a lower concentration of dissolved salts than is present in the cell's protoplasm, water will flow into the cell and, unless it has a strong cell wall, it will swell and may burst. On the other hand, when a cell is surrounded by a hypertonic solution of higher salt concentration than that of the protoplasm, water will leave the cell, causing dehydration of the protoplasm. In order to maintain the correct osmotic relations between the cell and its environment, it must be bathed in an isotonic solution or one in which the amount of dissolved salts is approximately equivalent to that inside the cell. Whether the effect of various salt concentrations on bacteria is entirely one of unfavorable osmotic pressure is unknown, but most bacteria die in distilled water and their growth is inhibited in media of relatively high salt content. However, a small amount of various salts in the medium is stimulating to bacterial growth. Traces of such salts are furnished by natural foods such as meat extracts or they may be added to the medium.

**Hydrogen Ion Concentration or pH.** Distilled water dissociates into an equal number of free hydrogen ( $H^+$ ) and hydroxyl ( $OH^-$ ) ions, and it has therefore, a neutral reaction. One liter of pure water contains 0.000,000,1 gm. of hydrogen ions, or a hydrogen ion concentration of  $1/10,000,000$  mols per liter. This figure may be expressed as  $1 \times 10^{-7}$  and the hydrogen ion concentration is said to be pH 7. The symbol pH is defined as the logarithm of the reciprocal of the hydrogen ion concentration. It provides a convenient way of expressing



nity or acidity. A neutral solution has the same hydrogen ion concentration as pure water and its  $pH$  value is 7. An acid solution has a higher hydrogen ion concentration than pure water and its  $pH$  is less than 7. Conversely, since an alkaline solution has a lower hydrogen ion concentration than pure water, its  $pH$  is greater than 7. The lower the  $pH$  (below 7) the more acid, and the higher the  $pH$  (above 7) the more alkaline the solution. Most bacteria like a neutral medium. Yeasts and molds prefer foods with a slightly acid reaction. The original composition of ingredients in media containing meats or meat products is generally slightly acid, and the hydrogen ion concentration must be adjusted to the neutral point or to a  $pH$  optimal for growth of a particular organism.

**Adjusting the Reaction of Culture Medium.** To determine the reaction, indicator solutions which change color near the neutral point are useful. From a list of indicators and their  $pH$  ranges given below, it will be noted that bromothymol blue, which is yellow in acid solutions, blue in alkaline solutions and green around  $pH$  7, is a good one for this purpose. A 5 ml. sample of the medium is measured into a test tube containing 20 ml. of distilled water and 10 drops of the indicator (a 0.04 per cent aqueous solution of bromothymol blue). If the reaction is acid, N/20 NaOH is added from a burette or pipette and mixed with the diluted medium until a grass green color appears, or until it matches the color of a comparable sample of a standard buffered solution of known and fixed  $pH$ . The amount of N/20 NaOH used in this titration multiplied by 10 is the amount of normal NaOH which should be added to each liter of the medium. After thorough mixing and reheating the  $pH$  is again determined to insure the proper reaction.

### COLOR CHANGE AND $pH$ RANGE OF INDICATORS

INDICATOR	COLOR CHANGE	$pH$ RANGE
Methyl red	red—yellow	4.4—6.0
Brom cresol purple	yellow—purple	5.2—6.8
Brom thymol blue	yellow—blue	6.0—7.6
Phenol red	yellow—red	6.8—8.4
Phenolphthalein	colorless—red	8.5—10.5
Litmus	red—blue	4.5—8.3

**Preparation of Common Culture Media.** Nutrient broth is the base or most commonly used media. It may be enriched with other nutrients and it may be made into a liquefiable solid by the addition of either gelatin or agar. The composition of nutrient broth is as follows:

Beef extract .....	3.0 gm.
Peptone .....	5.0 gm.
Distilled water .....	1000.0 ml.

Nutrient gelatin has the same composition as nutrient broth plus 10 per cent gelatin.

Nutrient agar contains 1 liter of nutrient broth plus 15 gm. agar.

Carbohydrate broths are prepared by adding 0.5 per cent of the required sugar or other carbohydrate to nutrient broth. An indicator may also be included to show changes in the pH resulting from acids produced from the carbohydrate by organisms growing in the medium. Phenol red, brom cresol purple and bromothymol blue are suitable, and 1 ml. of a 1.6 per cent alcoholic solution of one of these indicators is added to each liter of carbohydrate broth. Special sterilization methods (see directions below) are necessary to prevent decomposition of the sugars and polysaccharides. The final reaction of the broth for cultivation of most bacteria should be pH 7 to 7.2.

Sugar or starch agar is produced by dissolving 15 gm. agar and 10 gm. of the desired carbohydrate in 1 liter of nutrient broth.

General directions for preparing culture media are as follows:

+

1. Measure and mix ingredients.
2. Heat to dissolve ingredients, stirring frequently. Boiling is required in the case of media containing agar, but gelatin medium should not be boiled.
3. Make up loss of evaporation by adding distilled water.
4. Titrate and adjust reaction to the desired pH (generally about pH 7.5 since sterilization causes a slight increase in acidity).
5. Reheat medium to the boiling point, stir thoroughly and check reaction.
6. Broths should be filtered through filter paper, agar and gelatin media through moist absorbent cotton, to clarify.
7. Dispense into appropriate tubes or flasks and plug with nonabsorbent cotton.
8. Sterilize by autoclaving at 121° C (15 lbs. pressure) holding tubes packed in buckets for 20 minutes and flasks containing 1 liter amounts for 30 minutes. Sugar media for fermentation tests should be autoclaved at 15 lbs. pressure for 15 minutes only, or such media may be prepared by adding the carbohydrate solution which has been previously sterilized by filtration to a sterile broth base.

**Special Media for Parasitic Organisms.** Infusion media and other media rich in animal fluids or tissues are superior to meat extract media for the cultivation of certain fastidious parasitic organisms.

**Meat infusion broth** is made by steeping 500 gm. of finely ground fresh lean meat, generally veal or beef, in 1 liter of distilled water. No heat is applied but the infusion is allowed to stand overnight in the refrigerator. Then the supernatant of fat is removed, the liquid is expressed through a muslin cloth and it is made up to 1 liter with distilled water. The infusion is boiled for 1/2 hour, the 1 liter volume is then restored with distilled water, and 20 gm. peptone and 5 gm. sodium chloride are dissolved in the infusion by further heating. After the reaction is adjusted to pH 7.6-7.8, the medium is again boiled for 1/2 hour and cooled before filtering through paper. Directions for adjustment of reaction and sterilization are the same as those previously described.

**Meat infusion agar** is produced when 15 gm. agar are dissolved in 1 liter of infusion broth. The agar medium is filtered through absorbent cotton and sterilized as usual.



Blood agar is generally made from meat infusion agar containing 20 gm. per liter. Five to 10 ml. defibrinated sterile blood are mixed with 100 ml. and cooled (45° C) agar just before pouring plates or dispensing into tubes for blood agar slants.

Chocolate agar is made by adding from 5 to 10 per cent blood to melted meat infusion agar or nutrient agar and heating the mixture to 75° C until the blood begins to coagulate and turns a brown or chocolate color. When the medium has been cooled to about 45° C it may be poured into plates or dispensed into tubes for slants. Chocolate agar is especially useful for the cultivation of certain pathogenic bacteria such as *Hemophilus influenzae*.

Serum, ascitic fluid and egg media are used to grow many parasitic bacteria. Instead of blood, a small quantity of serum or ascitic fluid may be added to the medium, or the quantity of animal proteins added may be sufficient to solidify the medium when it is heated, as is the case in Löffler's serum medium and several varieties of egg media including Petragnani's medium (see Appendix.)

**Cultivation in Living Tissues.** Even the rich animal foods described above do not satisfy certain parasitic microorganisms which require fresh or living tissues. Parts of recently excised organs, bits of sterile rabbit kidney, for example, embedded in the medium may suffice; but, if this fails, lifeless concoctions may be abandoned entirely and living tissues provided. Thus, many agents of disease, notably filtrable viruses and rickettsiae, multiply in actively growing cells removed from the developing chick embryo and maintained in tissue cultures, or they are introduced directly into the fertile egg. Finally, pathogenic microorganisms may be grown in the body of a susceptible laboratory animal.

**Synthetic Media.** Media of known chemical composition are termed synthetic media. Pains-taking investigation is gradually revealing the exact combination of compounds necessary to grow certain bacteria, yeasts and molds. Some free-living microorganisms vie with the green plants in their ability to live on simple organic substances occurring naturally in soil and water. Their synthetic media are correspondingly simple. Even for certain parasites previously grown only on complex media or in laboratory animals satisfactory synthetic media have now been formulated. These contain a variety of chemically pure substances such as mineral salts, sugars, amino acids and traces of accessory growth factors including several members of the vitamin B complex. Theoretically, it should be possible to grow even the most fastidious organism on synthetic culture media if its nutritional requirements were known.

**Selective and Differential Media.** No inhibitory chemicals must be present in a satisfactory culture medium, but different microorganisms possess varying degrees of tolerance to such substances. This is particularly true with respect to the dyes: one concentration of gentian violet, brilliant green or basic fuchsin will inhibit some organisms and not others. This effect is related to the

staining properties of the cells, the gram-positive bacteria generally being more sensitive to the inhibiting action of the dyes than the gram-negative organisms. Molds and yeasts can grow in much more acid media than most bacteria. Advantage is taken of such facts in the preparation of selective media which allow certain organisms to grow and suppress the development of others.

Furthermore, media may contain substances which cause the growth of one kind of organism to appear different from that of another, and thus the identification of a particular species occurring in a mixture is facilitated. Litmus lactose agar will differentiate between lactose-fermenting and nonfermenting types. Only bacteria which produce hydrogen sulfide will blacken lead acetate agar. Endo's agar and eosin-methylene blue agar are lactose, dye-containing, selective media which permit growth of the gram-negative bacilli of the colon-typhoid-dysentery group and prevent multiplication of most other intestinal bacteria. Colonies of the colon bacillus are dark red on Endo's agar and dark purple with black centers on eosin-methylene blue agar due to fermentation of the lactose, while colonies of the pathogenic members of the group, being nonlactose fermenters, remain uncolored. Many similar selective and differential media, valuable in isolating and identifying microorganisms, are available.

**Dehydrated Culture Media.** Desiccated media which require only the addition of water and sterilization may be purchased from commercial laboratories. These ready-made preparations are especially valuable where small amounts of a medium are needed or where it is infrequently used, in complicated media whose ingredients may not be stocked and whose preparation calls for special practice, and in preventing variation in the composition of different batches of the same medium.

## CULTIVATION METHODS

Cultivation methods are useful in increasing the numbers of microorganisms in a given specimen, in separating different kinds of microorganisms, and in providing important clues to their identification. Cultivation often permits the isolation of bacteria from material in which they are too infrequent to be discovered by direct microscopic examination. If their growth requirements are satisfied, even a few bacteria will multiply into an enormous population and microscopic studies can be made of these cultures. Even if there are abundant bacteria present in a specimen, they may have no outstanding morphological or staining properties by which the bacteriologist can recognize them. In fact, the positive identification of bacteria with few exceptions necessitates a study of what they do, a study of their behavior in various kinds of culture media and sometimes, in laboratory animals.

To identify an organism by its cultural and physiological properties it is necessary to separate that organism from others and grow it alone in pure culture. Isolation in pure culture is essential for the recognition of previously described forms, for the discovery and classification of new species and for the determination of the causative agents of infectious disease. The most commonly



method of quantitative bacteriology, *i.e.*, the viable count, also depends on cultivating bacteria and counting those which can multiply on artificial media. **Methods of Inoculating Tubes of Culture Media.** The way microorganisms are introduced into a culture medium depends on the nature of the inoculum and of the culture medium. The inoculum is the material which contains the microorganisms and which is introduced into the sterile nutrient medium. Organisms are transferred from one tube of liquid medium to another with a wire loop. Both tubes are held in the left hand (unless the person is left handed) with the butts of the tubes held in the palm of the hand by the thumb of the hand so turned that the operator can see the contents of both tubes (Fig.

The mouths of the tubes should extend far enough beyond the finger tips so that the fingers are not burned when the ends of the tubes are flamed. The tubes should lie as horizontally as possible without wetting the cotton plugs. Thus, the chance of aerial contamination is greatly reduced. Just before inoculating the medium, the cotton plugs are loosened, and the inoculating needle, held in the right hand, is flamed

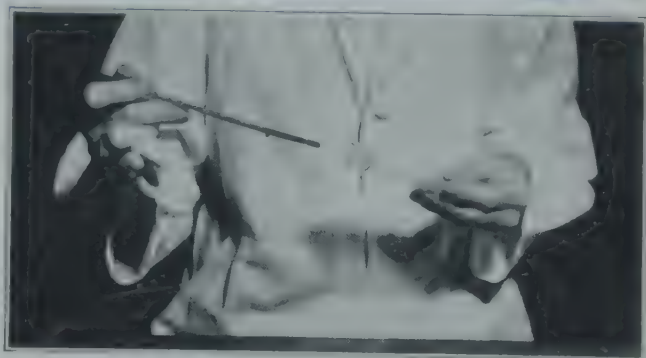


Fig. 103. Transferring a culture from one tube to another. Note the way the inoculating needle, test tubes and two cotton plugs are held.

the entire wire glows red. Immediately after allowing the needle to cool without touching anything, the cotton plugs are removed by the fingers of the left hand not engaged in holding the needle, the lips of the tubes are passed through the flame, and a loopful of the inoculum is transferred to the sterile tube as speedily as possible. Once more the mouths of the tubes are flamed, the cotton plugs are quickly but securely replaced, and the needle is again sterilized. The same procedure is followed in inoculating a slant of solid medium except that the loopful of inoculum is drawn over the slant without breaking its surface. A straight needle is used to stab deep tubes of solid media such as nutrient agar and lead acetate agar. **Shake cultures** are made by mixing the inoculum in deep tubes of cooled melted solid or semisolid medium. When the inoculum is a liquid culture, but microbial growth of a slant or plate culture, a small amount can be removed and transferred by either a straight or looped needle. Specimens from patients (blood, pus, sputum and the like) can also be inoculated into tubes of media by sterile inoculating needles, swabs, capillary pipettes or ringes.

**Pour Plates.** The object of the plate culture is usually to obtain isolated colonies of bacteria. A colony is a visible mass of microbial growth on solid culture medium resulting, theoretically, from the reproduction of a single microorganism. Proper inoculation of plates must somehow separate the bacteria of the inoculum and deposit them in fixed, isolated positions in or on the solid

medium. Obviously, the inoculum must not contain too many organisms, and hence it is advisable in the pour plate method to make plates from several dilutions of the specimen. A loopful of the material to be plated is inoculated

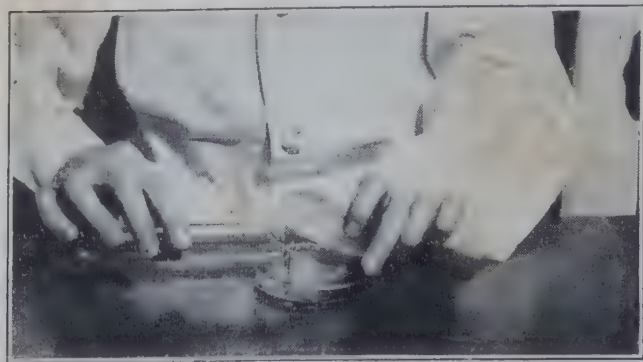


Fig. 104. Method of pouring a plate. The cotton plug may be held as shown or may be grasped between the ring and little fingers.

into a tube of sterile saline and after thorough mixing, a loopful of this dilution is introduced into a second tube, and so on until several dilutions have been made. A tube of melted agar is inoculated from each of these dilutions, or dilution may be made directly through a series of tubes of melted agar. In either case the melted agar, cooled to about  $45^{\circ}\text{C}$ , is inoculated with a loopful of the dilution, the tube is rotated to assure even distribution of the organisms, and the medium is poured into the culture dish. In this operation contamination is avoided by flaming the mouth of the tube before pouring the medium, by lifting the cover of the plate at one point just high enough and long enough to admit the end of the tube for pouring the medium, and by speed of action. The plate is gently rotated and tilted to spread the still fluid medium in an even film over its surface and is then set aside to harden in a level place. As soon as the medium is firm, the plate is inverted to prevent any moisture of condensation from dropping from the cover and spreading the bacteria from their fixed positions. After suitable incubation surface, subsurface and bottom colonies appear throughout the medium (Fig. 105).

**Streak Plates.** The best way to learn bacteriological techniques is to watch a good bacteriologist demonstrate the "tricks of the trade." This is especially true in

acquiring the knack of making satisfactory streak plates. The object of the streak plate, like that of the pour plate, is to grow isolated colonies, but here only surface colonies will develop. Separation of the organisms may be

into a tube of sterile saline and after thorough mixing, a loopful of this dilution is introduced into a second tube, and so on until several dilutions have been made. A tube of melted agar is inoculated from each of these dilutions, or dilution may be made directly through a series of tubes of melted agar. In either case the melted agar, cooled to about  $45^{\circ}\text{C}$ , is inoculated with a loopful of the dilution, the tube is rotated to assure even distribution of the organisms, and the

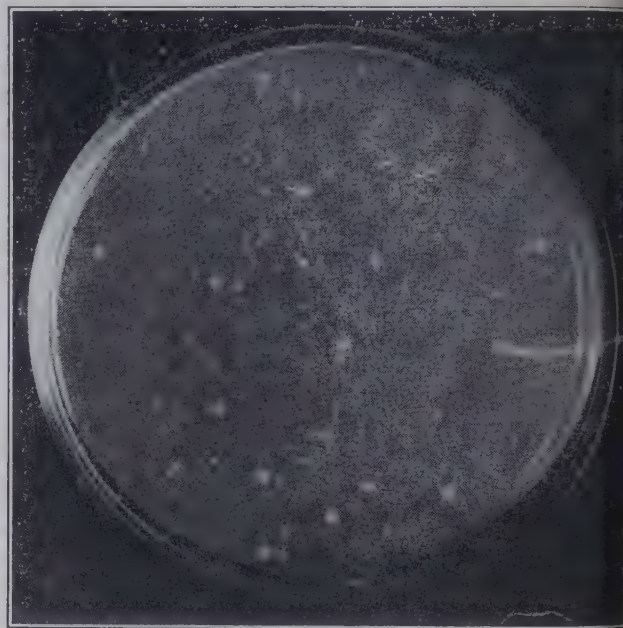


Fig. 105. Photograph of a pour plate culture of *Staphylococcus albus*. Note the larger, surface colonies and the smaller, lens-shaped, subsurface colonies.



achieved by drawing the inoculating needle bearing the inoculum lightly over the surface of hardened agar or other medium. The inoculum is first touched to the medium at one side of the plate close to the glass, and, regardless of the pattern followed in streaking the plate, it is then spread out in a series of lines from this spot. Usually the first streaks plant many bacteria close together and result in solid lines of growth, but with each successive streak fewer organisms are left behind, until there is sufficient space between neighbors to allow the development of isolated colonies (Fig. 106). To this end it is necessary to start

with a small inoculum and to cover the entire plate with many streaks leaving no "dead space" without breaking the surface of the medium. Generally the streaking is done with a bent or looped needle, but a loopful of the material to be plated is often too large an inoculum. One way of reducing the number of bacteria in the inoculum besides taking less of it is to sterilize the needle immediately after inoculating a small portion of the plate and then to streak the remainder of the plate, starting from the inoculated area, with the heated sterile needle. The same procedure is frequently followed when a swab is used to transfer a specimen to one spot on a plate



the specimen is streaked out with a sterile inoculating needle. To assure the formation of well isolated colonies it may be necessary to dilute the specimen before it is plated, or to continue streaking a second plate from the first. Though the streaking must be done carefully and according to some plan, it must be performed speedily with the cover of the plate held directly over the agar to prevent contamination. All plates should be inverted immediately after inoculation and during incubation.

**Anaerobic Cultivation.** Some bacteria known as **anaerobes** cannot live in the presence of atmospheric oxygen. Quite an assortment of devices have been produced for the purpose of cultivating these organisms but the principle underlying each is the same, *i.e.*, the elimination of free oxygen or the reduction of the medium. The culture medium is boiled before inoculation to drive out dissolved oxygen. Deep tubes of certain media allow anaerobic growth far below the surfaces. Many anaerobic culture media contain reducing substances which are efficient in using up oxygen. Chopped fresh meat, brain or other tissues,

sodium thioglycollate, cysteine and reduced glutathione are such substances. Chemicals, for example, pieces of self-igniting phosphorus or alkaline solutions of pyrogalllic acid, which absorb free oxygen, may be employed in a closed vessel containing the cultures. Anaerobic jars large enough to hold several plates or tubes of cultures have been devised from which oxygen can be eliminated by chemical and mechanical means. They can be evacuated of air and refilled with hydrogen or some other inert gas, and since carbon dioxide stimulates the growth of many anaerobes (as it does that of other organisms) some of this gas may be introduced

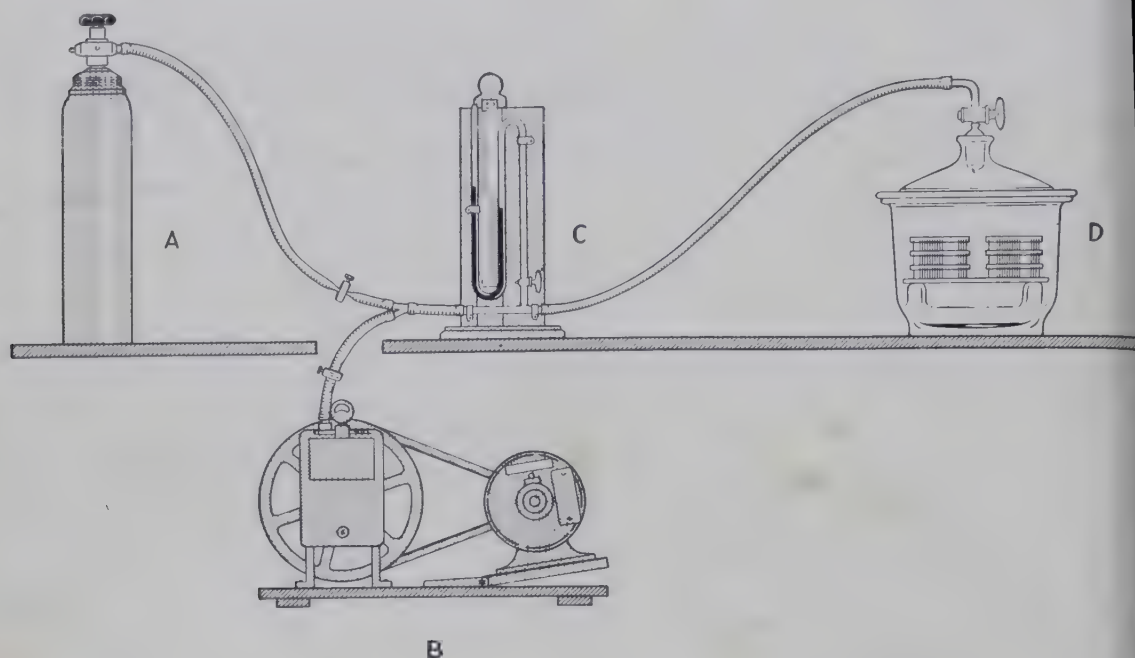


Fig. 107. Apparatus for evacuating anaerobic jar and supplying carbon dioxide for the cultivation of anaerobic bacteria. (Modification of Dock's method.)

A, Carbon dioxide tank; B, vacuum pump; C, mercury manometer; D, desiccator jar containing Petri plates and, at the bottom, an alkaline solution of pyrogalllic acid.

duced into the oxygen-free atmosphere (Fig. 107). Lastly, a variety of materials and mechanisms, among them sterile oil, vaseline, paraffin and constricted tubes with marble plugs, has been recommended for sealing the cultures away from the air or to stop convection currents. Combinations of two or more of these procedures are commonly used in one anaerobic method. Since it is impossible to give the details of all these methods, only those most likely to be used by the student will be described.

The **shake culture** is one of the simplest for growing anaerobes. Deep tubes of semisolid dextrose infusion (1 per cent) agar are boiled in a water bath for 10 to 20 minutes. After rapid cooling to about  $45^{\circ}\text{C}$ , the agar is inoculated by a loop or pipette submerged to carry the inoculum through the medium to the bottom of the tube. Gentle rolling of the tube between the palms of the hands helps to distribute the bacteria. Successive transfers from one tube to a second



a third will increase the chances of securing isolated colonies. Tubes are incubated in an upright position and anaerobic colonies develop deep in the medium. **Anaerobic slant cultures** may be made by applying Buchner's method of oxygen absorption by alkaline solutions of pyrogalllic acid (Fig. 108). Dextrose is boiled and quickly cooled to form a short slant. Inoculation is made by stabbing the butt and streaking the surface of the slant. The free oxygen in the tube is removed and the tube is sealed in the following way. The cotton plug is blown off flush with the mouth of the tube and the remaining medium is pushed down until it almost reaches the agar. The tube is plunged, mouth down, into pyrogalllic acid powder to fill the space above the plug. About 2 ml. of 5 per cent  $\text{Na}_2\text{HPO}_4$  (or  $\text{Na}_2\text{CO}_3$ ) solution are added, a rubber stopper is firmly inserted and the tube is inverted. The stoppered tube may be dipped into melted paraffin as a further protection against leaks. After incubation, when the culture is required, a fresh cotton plug should be substituted for the original one.

The **Spray anaerobic culture dish** is one of several modifications of the Petri plate (Fig. 109). One section of the dish is deep with a ridge dividing it into two shallow compartments planned to hold pyrogalllic acid on one side and an alkaline solution ( $\text{NaOH}$ ) on the other without any contact between the two chemicals until the dish is tipped. The other section designed to hold the agar medium is like the bottom of a regular Petri plate. Plates may be made of freshly boiled dextrose or other agar frequently enriched with the addition of blood or serum, and may be inoculated either by the pour or streak method. After inoculation the dish is inverted and the acid and alkali are placed in their respective compartments. The dish is sealed usually with oil, paraffin or plastocene and then tilted to allow contact of the agar with oxygen-absorbing chemicals.

## QUANTITATIVE BACTERIOLOGY

There are several ways of estimating the number of bacteria in a given amount of material. The **direct microscopic count** has been described under microscopic methods (Chapter 9). A quantitative method known as the **reductase estimate** or reductase test is used in reckoning the relative numbers of bacteria in milk (see Chapter 43). **Estimate by Dilution in Liquid Media.** It is possible to make an approximate calculation of the numbers of bacteria in a sample by diluting a unit amount (milliliter of liquids; 1 gram of solids or semisolids) in a series of broth tubes

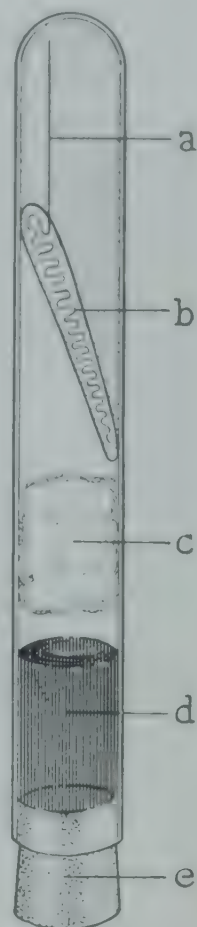


Fig. 108. Anaerobic slant culture, Buchner's method. a, Slab inoculation; b, line of inoculation on agar slant; c, cotton plug; d, oxygen-absorbing alkaline solution of pyrogalllic acid; e, rubber stopper.

in which each succeeding tube is diluted 10 times more than the one before. Thus, if 1 ml. of the sample is transferred to 9 ml. of sterile nutrient broth the becomes a 1:10 dilution of the original material. After thorough mixing, 1 ml. from Tube 1 is added to 9 ml. of sterile broth in Tube 2, making a 1:100 dilution, and so on until the last dilution is so high that the final tube shows no growth after incubation. If, for example, the tube which remains sterile is the 1:1,000,000 dilution and the highest dilution which produces growth is the 1:100,000 tube, it is logical to deduct that at least 100,000 bacteria were present

in the original 1 ml. sample. Several replicates of the same sample must be run simultaneously and the results checked for any degree of accuracy.

**Plate Count.** Each bacterial colony is the result of the multiplication of at least one living bacterium. If a known amount of material, say 1 ml. of tap water, is thoroughly mixed with a tube of cooled melted agar and a pour plate is made, it follows that the number of colonies developing on the plate cannot be greater than the number of bacteria present in that amount of water. The number of colonies is an indication of the number of bacteria in the



Fig. 109. The Spray anaerobic culture dish. (From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Appleton-Century-Crofts, Inc.)

sample which were alive and capable of growth under the cultural conditions provided. The colony count is not identical with the actual number of bacteria present in the sample. Rather it is always an underestimate because (1) some bacteria in the sample may be dead, (2) some may not find the culture medium, the oxygen supply and incubation temperature compatible with growth and (3) undoubtedly some colonies develop from clumps of bacteria rather than from single cells despite vigorous shaking and dilution of the sample previous to plating. If the sample contains so many bacteria that the colonies developing on the plate are not well isolated and are too numerous for accurate counting, the sample must first be diluted and 1 ml. of these dilutions plated. One gram amounts of solids and semisolids must also be diluted in order to free the bacteria embedded in the material before plating. The actual concentration of bacteria in a given sample is, of course, unknown; but judging from past experience the bacteriologist assumes about how many organisms are present in each milliliter or gram and then proceeds to dilute accordingly. For instance, if a sample of milk contains 25,000 bacteria per milliliter, plates made from the following dilutions theoretically would yield these colony counts:



DILUTION	COLONY COUNT
Undiluted	25,000
1:10	2,500
1:100	250
1:1,000	25
1:10,000	2
1:100,000	less than 1

ously plates made from this undiluted milk and from the lowest dilution grow too many colonies to count; on the other hand, there is the danger of over-diluting. The ideal plate has between 30 and 300 colonies. After considering the probable density of bacteria in the sample, duplicate plates are made from



Fig. 110. Intraperitoneal injection of a mouse. (Wadsworth: *Standard Methods*, Wm. B. Saunders Co., and Kolmer and Boerner: *Approved Laboratory Technic*, 4th ed., D. Appleton-Century Co., Inc.)

for three different dilutions whose counts are most likely to approach these values. Thus, in the illustration above, duplicate plates of both the 1:100 and 1:1,000 dilutions would be made. After suitable incubation the colonies on each plate are counted. This colony count is multiplied by the dilution factor to arrive at the bacterial count indicated by each plate, and the final estimate of bacteria per milliliter (or gram) is made by comparing the bacterial counts resulting from all plates made from the same sample, averaging counts that are not more than 20 per cent apart. Fictitious accuracy is avoided by stating the final estimate in round numbers. Since the count method cannot possibly enumerate every bacterium, a final figure of 23,850 bacteria per milliliter gives a false impression and is stated as 24,000 per milliliter.

## ANIMAL INOCULATION

Experimental animals are inoculated (1) to discover the cause of an infectious disease, (2) to test the disease-producing power or virulence of a pathogen, (3) to obtain a concentration of or a pure culture of a pathogen when it is mixed with nonpathogens, (4) to grow agents of disease such as rickettsiae, filtrable viruses and fastidious bacteria which refuse to grow on laboratory media, and (5) to produce immune serum. It is desirable that the animals be small, easy to handle, cheap and adaptable to the living conditions of the laboratory. Guinea pigs, white



Fig. 111. Intravenous injection of a rabbit. (From Kolmer, J. A.: *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co.)

mice, white rats and rabbits have these qualifications and they are generally used, but they do not always fulfill the requirements of a satisfactory experimental animal, for they are not susceptible to all infectious diseases. A search for animals susceptible to certain human or animal infections has resulted in the use of a variety of animals, including monkeys, ferrets and canaries, while in some other diseases—for example, leprosy and typhoid fever—the search has thus far been unsuccessful. Not only must a suitable test animal be selected, but the microorganisms or their toxic products must be introduced into the animal by the proper route. The most common routes of inoculation are subcutaneous (just under the skin), intracutaneous (into the layers of the skin), intraperitoneal (through the body wall into the peritoneal cavity) and intravenous (into a vein). The inoculum is drawn into a sterile syringe and injected into the animal through a sterile hypodermic needle. The site of injection is prepared by removing the hair, cleansing the skin and applying a germicidal solution. Asepsis is followed not only in the inoculation but also in the subsequent removal of specimens and at postmortem examination.



# 12

## PURE CULTURE METHODS AND THE IDENTIFICATION OF BACTERIA

### ISOLATION OF BACTERIA

A culture containing two or more species of microorganisms is known as a mixed culture while one containing only one kind is a pure culture. In nature, pure cultures rarely if ever occur. An amazing variety of organisms live together in microbial communities in their natural habitats such as the soil, water, and parts of the body in contact with the environment. The study of these microbial communities and their cooperative and antagonistic actions is a fascinating and well-known subject, for microorganisms are generally studied in pure cultures. Much has been learned in this way that it is no wonder bacteriologists use the pure culture method routinely, almost exclusively, in trying to understand the structure and activities of microorganisms. The discovery of a method by which bacteria could be isolated and grown in pure culture was probably the greatest contribution to the science of bacteriology.

**Dilution Method.** Before Koch introduced gelatin and agar media, early bacteriologists practiced this tedious and often unsatisfactory plan. From a very crude culture containing a variety of organisms, a minute portion was removed and transferred to a second tube of sterile liquid medium. From this second tube a third was inoculated, and so on until somewhere in the series there occurred, after incubation, a tube of medium that remained sterile. The preceding tube, *i.e.*, the last culture medium of the series showing signs of growth, was supposed to be a pure culture. The disadvantages of this method are obvious. First, there is no certainty that only one bacterium or one kind of bacterium will be carried over into that last culture. Furthermore, even if the method succeeds in producing a pure culture, the operator has no choice as to the kind of organism he isolates. Instead, the bacterium which outnumbers all others in the original mixture or which is most favored by the conditions of cultivation is the one which will grow in the last, pure culture.

**Picking a Colony.** Proper inoculation of streak or pour plates separates the microorganisms in a specimen and plants them in fixed positions where they develop into isolated colonies. Theoretically, each colony is the result of the reproduction of a single cell, but probably a pair or a clump of sister cells often

becomes fixed at the same spot and starts a colony. If a colony has developed from a single organism or a single kind of organism, a bit of the colony inoculated into sterile medium should result, after suitable incubation, in a pure culture. Pure cultures are obtained routinely by this method. **Proof of purity** is made (1) by **microscopic examination** to ascertain that all the organisms are alike as to morphology and staining properties and that they resemble those of the parent colony; (2) by **plating** to see that all colonies are alike and are similar to the parent colony.

**Single Cell Technique.** This technique is the latest advance in pure culture methods. With the aid of a special apparatus, a micromanipulator, attached to the stage of the microscope, the skilled operator can pick up a single bacterial cell with the tip of a capillary pipette. The bacteria of the original mixed culture are first widely dispersed in a liquid by dilution. A drop of this dilution is observed under a high magnification of the microscope, the desired cell is "sighted" and the operator, by means of the micromanipulator, directs the tip of the sterile pipette toward the cell. The pipette picks up the single cell by capillarity, removes it from the drop and deposits it into sterile culture medium. This delicate operation requires special training and the method is used only in work where the utmost precautions must be taken to assure purity of cultures or where the organisms cultivated must be the progeny of a single cell.

**Aids in Obtaining Pure Cultures.** A knowledge of the growth requirements or other characteristics peculiar to the microorganisms to be isolated may offer some clue helpful in fostering their development and suppressing that of others. Strict aerobes and anaerobes can be separated from each other by regulation of the oxygen supply. Since many organisms have different optimum temperatures, different incubation temperatures may be used to encourage the growth of some and to restrain others. If a mixture of a sporulating and non-sporulating bacteria is brought to a boil or heated sufficiently to destroy the vegetative forms, the spores alone remain viable. Selective media are of immense value in the primary cultivation of a highly contaminated specimen, for as previously explained, only certain organisms are allowed to grow and others are eliminated. Disease-producing microorganisms will grow when introduced into a susceptible laboratory animal, while free-living, saprophytic forms will not survive. Therefore, specimens containing mixtures of a pathogen and non-pathogens may be injected into an animal and after an appropriate interval the pathogen may be recovered in pure culture.

## IDENTIFICATION OF BACTERIA

**Systematic Study of a Pure Culture.** To learn the properties of a microorganism, to identify that organism and fit it into the classification of microorganisms a thorough and systematic study of a pure culture is required. A definite plan for such a study of bacteria has been prepared by a committee of the Society of American Bacteriologists and the results of the investigation may



recorded in a descriptive chart published by this organization. The plan seeks to discover the properties of the bacterium which are most likely to differentiate it from others. In other words, a systematic study of a bacterium reveals those characteristics which form the basis of the classification of all bacteria.

Bacteria are classified according to their (1) morphology, (2) physiology, (3) pathogenicity and (4) immunological reactions. Morphology is the study of form and structure; the first step in classifying a bacterium is to observe its appearance under the microscope and its growth in culture media. Larger organisms are subdivided into groups largely on the basis of morphology, but the use of their lack of morphological variety bacteria cannot be classified by morphology alone. The physiology of bacteria, *i.e.*, their cultural and metabolic properties, must also be determined. It is necessary to find out what they do in different physical and chemical environments. Morphologically similar bacteria may be entirely dissimilar in their abilities to grow at certain temperatures, to utilize various substances as foods, to produce certain biochemical changes in culture media and to exhibit a number of other distinct behavior characteristics. Pathogenicity, which is the ability of a microorganism to produce disease, is a property of some bacteria. To test the pathogenicity of a microorganism it must be injected into a susceptible animal by a suitable route. The identification of bacteria does not require animal inoculation, but where certain bacteria resemble pathogens morphologically, culturally and biochemically, pathogenicity must be determined. It may be impossible to separate very closely related bacteria, especially pathogenic bacteria, on the basis of any of the above-mentioned properties. They may appear to be alike in all respects, but the chemical composition of their cells may be slightly different. This difference in perhaps only one chemical constituent can often be detected by immunological methods which will be explained in a later section.

Some of the morphological and physiological characteristics important in the identification of bacteria may be outlined as follows: \*

#### 1. Morphology

1. Gross morphology (especially of colonies with respect to size, shape, texture, color, etc.)
  - (a) on nutrient or infusion media
  - (b) on special media
2. Microscopic morphology including
  - (a) size, shape and grouping of the cells
  - (b) presence or absence of spores
  - (c) motility
  - (d) presence or absence of capsule
  - (e) staining reactions

#### 3. Biochemical reactions

1. Action on carbohydrates including the fermentation of sugars (dextrose, lactose, sucrose, etc.) and the hydrolysis of starch
2. Liquefaction of gelatin

Adapted from Jordan and Burrows, *Textbook of Bacteriology*, 14th ed., W. B. Saunders, Philadelphia, 1945.

3. Formation of indole
4. Production of hydrogen sulfide
5. Reduction of nitrate to nitrite
6. Hemolysis on blood agar
7. Other special biochemical tests

**Cellular Morphology.** Stained smears are examined for shape of cells, grouping, spore formation, presence of capsules, number and arrangement of flagella and staining reactions. Special methods must be used to demonstrate flagella. Capsules may show up in ordinary preparations, but negative staining and others to color the background or the capsule may be necessary. Any staining procedure that tints the vegetative cells will reveal spores, although special spore stains are available. Staining irregularities in the cells due to vacuoles and granules are apparent with most stains. Gram-positive and gram-negative bacteria are inherently different and the reaction of young cells to the Gram stain is a primary distinguishing characteristic. Pleomorphism, or variation in cellular morphology in an actively growing pure culture, is a trait of some species. Cells from a young culture are studied for evidence of motility in a hanging drop preparation.

**Gross or Cultural Morphology.** The gross morphology of bacteria is studied in plate, slant, broth and stab cultures. Different species characteristically produce different population patterns. **Colonies** on gelatin and agar plates are described as to their **form, elevation, topography or surface character, margin, pigment production and density**. **Agar slant or stroke** culture examination includes **amount of growth, odor and consistency** as well as **form and chromogenesis**. **Broth cultures** of some species are **cloudy throughout** due to an even suspension of the cells. Others, due to differences in oxygen relations and other physical phenomena, form **zones of cloudiness**, develop a **sediment** at the bottom of the tube, or concentrate at the surface of the liquid as a **pellicle** (scum) or **ring** of growth. Since bacteria may or may not digest and liquefy gelatin, gelatin stab cultures incubated at temperatures below the melting point of the medium may give information regarding either the **form of growth along the line of inoculation** in the undigested gelatin or the **form of liquefaction** extending from the line of stab. The value of these morphological and cultural properties rests in the fact that despite variation they are remarkably constant in each species of bacterium and readily allow recognition of typical forms of growth. Figures 112, 113 and 114 illustrate various types of cultural morphology and commonly used descriptive terms.

**Action on Blood Agar.** Bacteria growing on blood agar frequently produce total or partial destruction of the red blood cells (**hemolysis**) in the medium. Certain types clear the red color of the medium by complete hemolysis to a colorless zone around their colonies, some colonies produce a hazy, greenish zone of discoloration in which there may or may not be partial hemolysis, and others effect no change on blood agar. Reaction on blood agar plates is a particularly important criterion for separation of the great group of streptococci (Fig.



Those which produce greenish discoloration of the blood agar are known as *alpha* ( $\alpha$ ) streptococci, those which clear the medium or cause complete hemolysis are named *beta* ( $\beta$ ) streptococci, and those which are inactive on blood agar or non-hemolytic are referred to as *gamma* ( $\gamma$ ) streptococci. Hemolysis on blood agar is a property of many kinds of free-living as well as parasitic bacteria.

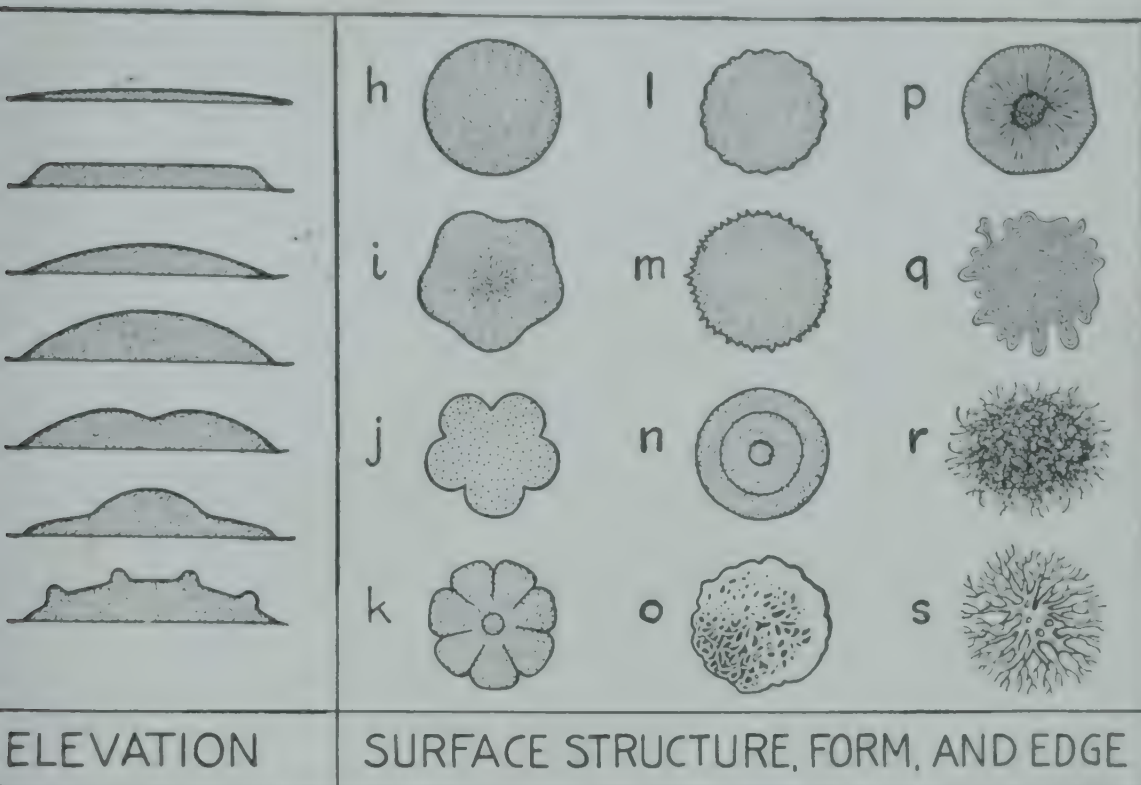


Fig. 112. Types of bacterial colonies. Elevation: a, flat or effuse; b, raised; c, low convex; d, high convex (dome-shaped or pulvinate); e, umbilicate; f, umbonate; g, verruciform. Surface structure, form and edge: h, circular, amorphous, edge entire; i, regular, edge undulate; j, irregular, granular, edge lobate; k, regular, radiate, edge entire; l, regular, edge crenated; m, regular, edge dentated; n, regular, concentrically ringed, edge entire; o, irregular, rugose; p, regular, mycelioid; q, irregular, curled, edge undulate; r, irregular, filamentous; s, irregular rhizoid.

From Belding and Marston: *A Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)

**Temperature Relations.** Four critical temperatures, characteristic of each species of bacterium, are determined in a systematic study. Pure cultures of the bacterium are incubated at various temperatures to discover its **optimum temperature** and the range of temperatures through which it can grow. The lowest degree supporting growth is the organism's **minimum temperature**, and the highest is its **maximum temperature**. Twenty-four hour nutrient broth cultures are exposed to increasing degrees of heat for a fixed time, say 10 minutes, to discover the resistance of the organism to heat. The lowest temperature which sterilizes the culture exposed to it for the stated period of time is said to be the **thermal death point** of that bacterium.

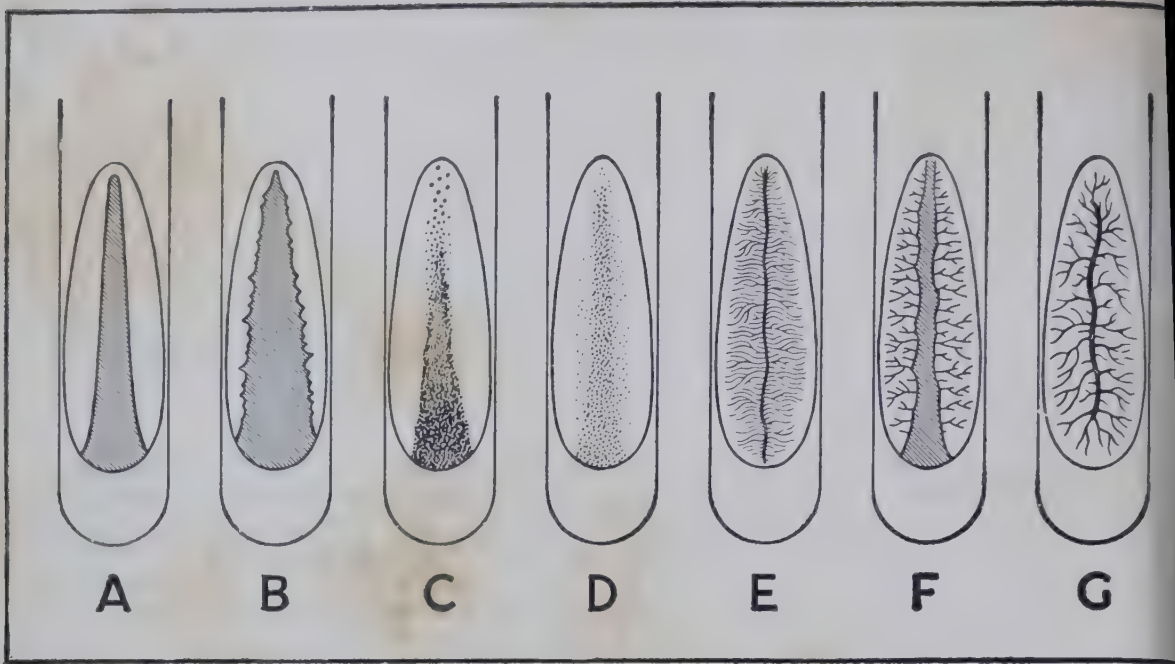


Fig. 113. Types of growth along line of inoculation in agar slant cultures. A. Filiform; B, echinulate; C, beaded; D, effuse or spreading; E, plumose; F, arborescent; G, rhizoid. (From Belding and Marston: *A Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)

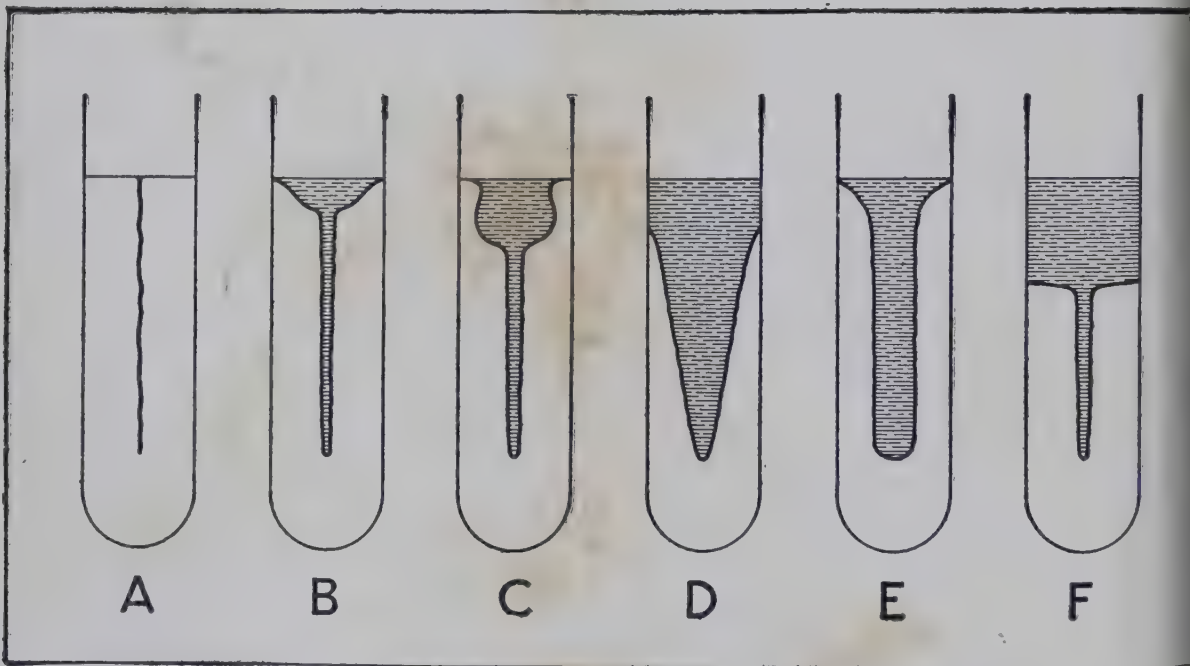


Fig. 114. Types of gelatin liquefaction. A, Filiform growth without liquefaction; B, crateriform; C, napiform; D, infundibuliform; E, saccate; F, stratiform. (From Belding and Marston: *A Textbook of Medical Bacteriology*, D. Appleton-Century Co., Inc.)



Bacteria may be grouped according to their growth-temperature relations. Those growing best or only at low temperatures are described as psychrophilic (cold-loving), those having a moderate optimum temperature, such as that of the human body ( $37^{\circ}\text{C}$ ), are known as mesophilic, and those preferring or requiring a relatively high temperature are termed thermophilic (heat-loving) bacteria. The following classification illustrates how the temperature ranges of the three groups may overlap. One should remember that the temperature range of an organism refers to the temperatures which support growth and that it is no indication of the organism's ability to survive more extreme degrees of heat and cold.

BACTERIA ACCORDING TO GROWTH-TEMPERATURE RELATIONS	CRITICAL TEMPERATURES			EXAMPLES
	MINIMUM	OPTIMUM	MAXIMUM	
Psychrophiles	$0^{\circ}\text{C}$	$4^{\circ}\text{--}10^{\circ}\text{C}$	$30^{\circ}\text{C}$	Many species of water bacteria. Bacteria spoiling foods held in cold storage
Mesophiles	$15^{\circ}\text{--}25^{\circ}\text{C}$	$25^{\circ}\text{--}40^{\circ}\text{C}$	$43^{\circ}\text{C}$	Most bacteria
Thermophiles	$25^{\circ}\text{--}45^{\circ}\text{C}$	$50^{\circ}\text{--}55^{\circ}\text{C}$	$85^{\circ}\text{C}$	Certain bacteria growing in soil, water, hot springs

Some organisms, particularly certain pathogenic bacteria, can grow only in a very narrow temperature range and these are named **microphiles**. Such a thermophilic bacterium is the gonococcus, with a minimum temperature of  $30^{\circ}\text{C}$  and a maximum temperature of  $38.5^{\circ}\text{C}$ .

**Oxygen Relations.** Aerobic and anaerobic pure cultures will indicate whether a bacterium can grow only in the presence of atmospheric oxygen (an obligate aerobe), whether it develops only in the absence of free oxygen (an obligate anaerobe), or whether it can adjust itself to either an aerobic or an anaerobic environment (a facultative organism). Bacteria which require an oxygen tension less than that of the atmosphere are known as **microaerophiles**.

**Food Requirements and Optimum pH.** Culture media which will grow one kind of bacteria may not be suitable for another. The reasons for these different food needs, like those of all other growth requirements, go back to differences in the nature and functions of the protoplasm in different bacterial species. Consequently, food preferences may be valuable in identifying bacteria. For example, since *Hemophilus influenzae* will grow on blood agar but not on ordinary nutrient agar, this trait is an important clue in deciding whether or not an organism is *H. influenzae*. The ability of an unknown organism to grow on a medium containing only inorganic compounds such as ammonium salts and carbonates greatly limits the possibilities. In the same way knowledge of the

relation of a bacterium to the reaction of the culture medium, its optimum  $pH$  and  $pH$  range, is an asset in determining its identity. Aciduric, *i.e.*, acid-tolerating bacteria, such as the lactobacilli, can be distinguished from nonaciduric forms by testing for this property.

**Action on Carbohydrates.** The ability of a bacterium to digest and ferment certain sugars, starch and other carbohydrates is a property as typical of that species as its morphology and considerably more useful in identifying many bacteria. Why different bacteria act differently on these foodstuffs will be explained later in the discussion of their nutrition. Pure cultures are grown in fermentation tubes of carbohydrate broths which contain an indicator. After incubation, which is usually complete in 48 hours but may have to be prolonged for two or three weeks in the case of a few slow fermenters, the cultures are examined for acid and gas production. One bacterium may ferment a sugar, causing both acid and gas to form in the culture (recorded often as AG); another may produce acid but no gas in the same sugar broth (A); and still a third kind may not attack the sugar at all (-). In the last instance the color of the indicator shows no lowering of the  $pH$  and there is no gas caught in the tube but the broth is cloudy with bacterial growth. Colonies growing on starch agar plates may or may not be able to hydrolyze starch to sugars. To test for this action the plate is flooded with iodine solution; hydrolysis of the starch is indicated by a colorless zone extending out from the colony, while the undigested starch in the medium reacts with the iodine to give a blue color.

**Action on Proteins.** Pure cultures of bacteria differ in their power to digest and putrefy proteins and in the products formed as a result of their putrefactive action.

**Peptonization (Proteolysis).** Proteins are insoluble in water; for example, casein is a protein and milk is opaque because the casein molecules are insoluble. However, when a bottle of milk stands until it is completely spoiled it becomes an almost clear, watery solution. What happens is that the large casein molecules are digested or broken down by the bacteria growing in the milk into molecules small enough to go into solution. Peptones are partially digested proteins which are soluble in water. The change in the physical state from a solid to a liquid is evidence that the protein has been hydrolyzed or digested at least to the peptone stage, and the liquefaction of proteins is spoken of as either **peptonization** or **proteolysis**. Some bacteria are more active in this respect than others. Tests for proteolytic activity are commonly carried out by inoculating pure cultures into **milk, gelatin, coagulated egg albumin and coagulated blood serum**. The changes produced in these media are characteristic of the species.

**Action on Milk.** Either litmus milk or purple milk (the latter containing the indicator brom cresol purple) is a valuable medium to learn about an organism's actions on both the carbohydrate (lactose) and the protein (casein) constituents of the milk. These changes are possible: (1) the indicator may show **acid reaction** due to fermentation of the lactose; (2) enough acid may



pigmented sulfur bacteria and the chemosynthetic action of the autotrophic bacteria have been described in a previous chapter (Chapter 6). Another example of chemosynthetic autotrophism will be given in the discussion of the nitrifying bacteria, *Nitrosomonas* and *Nitrobacter*, two soil organisms which obtain energy by oxidizing ammonia to nitrites and nitrites to nitrates, respectively (page 186). There are many border-line types of bacteria which can utilize organic food if it is available or they can manage on a strictly inorganic diet. Such bacteria are known as **facultative autotrophs** in contrast to those which can utilize only inorganic substances, the **obligate autotrophs**. It has been suggested that the obligate autotrophs may be the most primitive of the bacteria and possibly of all organisms, for they could live in a world in which no other forms of food existed. On the other hand, the synthetic powers of such autotrophs, building all cell substance as they do from simple inorganic compounds and elements, exceed those of other organisms and their biochemistry must be extraordinarily complex.

The bacteria are arranged according to their food requirements, a gradual transition from obligate autotrophs to fastidious heterotrophs is evident. This transition can be summarized in the following outline of their food needs.

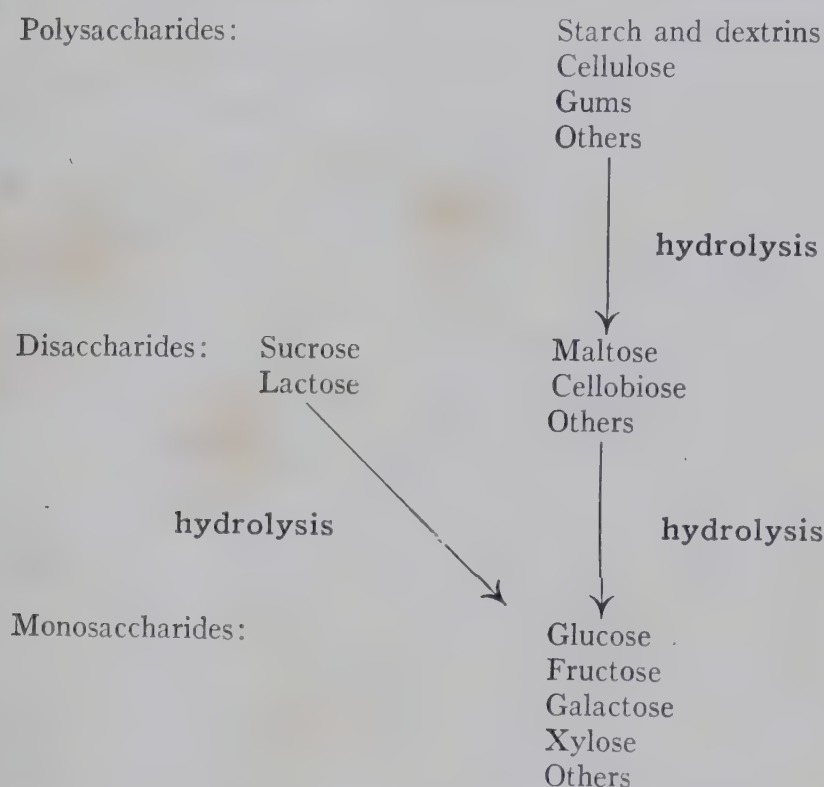
GROUP 1 Autotrophs	Carbon from $\text{CO}_2$ Nitrogen from inorganic sources Energy from inorganic oxidations or photosynthesis
GROUP 2 Facultative autotrophs and heterotrophs	Carbon from organic compounds Nitrogen from inorganic compounds Energy from organic compounds
GROUP 3 Fastidious heterotrophs	Carbon from organic compounds Nitrogen from amino acids Energy from organic compounds
GROUP 4 Non-fastidious heterotrophs	Carbon from organic compounds Nitrogen from a variety of amino acids Energy from organic compounds One or more accessory growth substances such as vitamins of the B complex

The nutritive requirements of the rickettsiae (and possibly the viruses) included here, they could be considered a fifth group comprising obligate, cellular parasites whose synthetic powers are so reduced that multiplication occurs only inside the living cells of a plant or animal host. The kinds of substances available to each species and how these substances are used once they are absorbed by the cells are determined by the enzymes the organism possesses. As in the case of the yeasts and molds, the greater the variety of digestive enzymes a bacterium secretes into its surroundings, the greater the range of food substances at its disposal and the better the chances are for that organism's survival.

## CARBOHYDRATE METABOLISM

**Digestion of Carbohydrates.** (By hydrolysis the exoenzymes of bacteria split complex carbohydrates, polysaccharides and disaccharides, into simple sugars, chiefly glucose, which can diffuse through the cell wall and come into contact with the protoplasm of the cell. The hydrolytic enzymes which effect the digestion of carbohydrates are highly specific.) If a pure culture of a bacterium is grown in a starch medium this polysaccharide will be of no use to the bacterium unless they secrete both amylase and maltase. The amylase hydrolyzes a complex starch molecule  $(C_6H_{10}O_5)_n$ \* to maltose  $(C_{12}H_{22}O_{11})$ , but only maltase can split maltose into two molecules of glucose  $(C_6H_{12}O_6)$ . In the same way a bacterium growing in a lactose broth could not ferment that disaccharide unless the enzyme lactase was present to digest it to the monosaccharides glucose and galactose. Some of the common carbohydrates and the products of their hydrolysis are outlined below in Table 6.

TABLE 6. DIGESTION OF CARBOHYDRATES †



The bacteria derive no benefit from such extracellular hydrolysis other than the transformation of complex food into a simple soluble form available for use.

**Utilization of Carbohydrates.** Since glucose is the most common end product of carbohydrate digestion, the sugar metabolism of the bacterial cell can be explained in terms of the utilization of this substance. Once in contact with the

\* The  $n$  after the formula means that the starch molecule is actually a multiple of  $C_6H_{10}O_5$ .

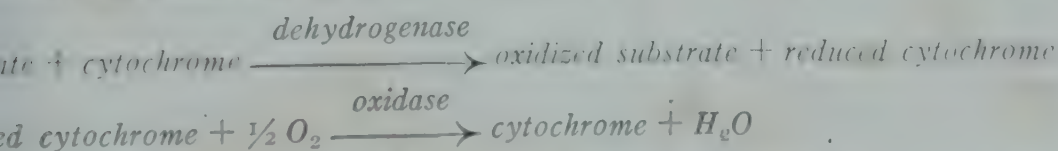
† Adapted from Henrici, *The Biology of the Bacteria*, D. C. Heath and Company, 2nd ed. New York, 1939.



As a glucose molecule may serve as a source of energy, it may be built into a substance or it may be changed into an insoluble carbohydrate, glycogen or starch and stored as reserve food. Systems of endoenzymes catalyze each reaction involved in these changes. The part played by the respiratory enzymes in the breakdown or dissimilation of glucose with accompanying release of energy is best understood at present.

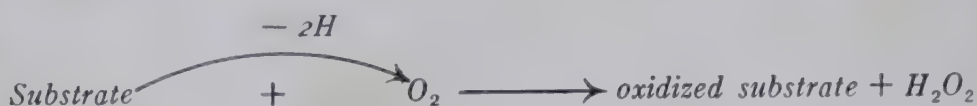
**Bacterial Respiration. The Dissimilation of Glucose.** Respiratory reactions are oxidations which release energy needed by the organism for the synthesis of cell substance and other activities. Until Pasteur's discovery of anaerobic respiration it was believed that the oxygen of the air always took part in such reactions and was essential to the life of all organisms. The phenomenon of "life without air" has since been explained by the study of anaerobic respiration and fermentation. To understand and compare aerobic and anaerobic respiration it is necessary to know something of the nature of the energy-releasing reactions. **Biological oxidations which occur in bacterial cells.**

A substance may be oxidized (1) by the addition of oxygen, (2) by the addition of hydrogen, or (3) by the loss of an electron (change in valence) without oxygen or hydrogen figuring in the reaction. Most biological oxidations are effected by the removal of hydrogen, *i.e.*, they are dehydrogenations catalyzed by enzymes known as **dehydrogenases**. The dehydrogenase removes the hydrogen from the substance being oxidized (the **hydrogen donator**) and allows its transfer to a substance, or one of a series of substances (**hydrogen carriers**), which then transfers it eventually to oxygen or to some other reducible substance (**hydrogen acceptor**). In aerobic respiration atmospheric oxygen is the hydrogen acceptor, while in anaerobic respiration other substances, such as products of glucose and protein decomposition, compounds like nitrates or even sulfur, iron and carbon may function in this capacity. The oxygen requirements of bacteria are determined then by their ability or lack of ability to utilize substances other than the oxygen of the air as hydrogen acceptors. Obligate aerobes must use oxygen, facultative anaerobes are endowed with alternative mechanisms which may employ oxygen or some other reducible substance, and in the case of the obligate anaerobes oxygen never serves as a hydrogen acceptor. Facultative anaerobes are not equipped with the respiratory pigment **cytochrome**, which transfers electrons to oxygen, nor with an **oxidase** enzyme that utilizes molecular oxygen as an electron acceptor. Almost all aerobic bacteria possess cytochrome and the proper oxidase to perform the final transfer of electrons to oxygen which results in the formation of reduced cytochrome and water.

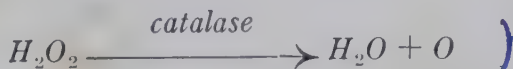


In the case of most aerobes and facultative bacteria growing aerobically certain substances other than cytochrome and its oxidase may also effect the final transfer of hydrogen to molecular oxygen and when this happens hydrogen peroxide

( $H_2O_2$ ) is commonly formed. Hydrogen peroxide formation may be represented by the following summary equation:



Peroxides are toxic to bacteria and most aerobic and facultative bacteria are protected by an enzyme, **catalase**, which decomposes hydrogen peroxide into water and oxygen:



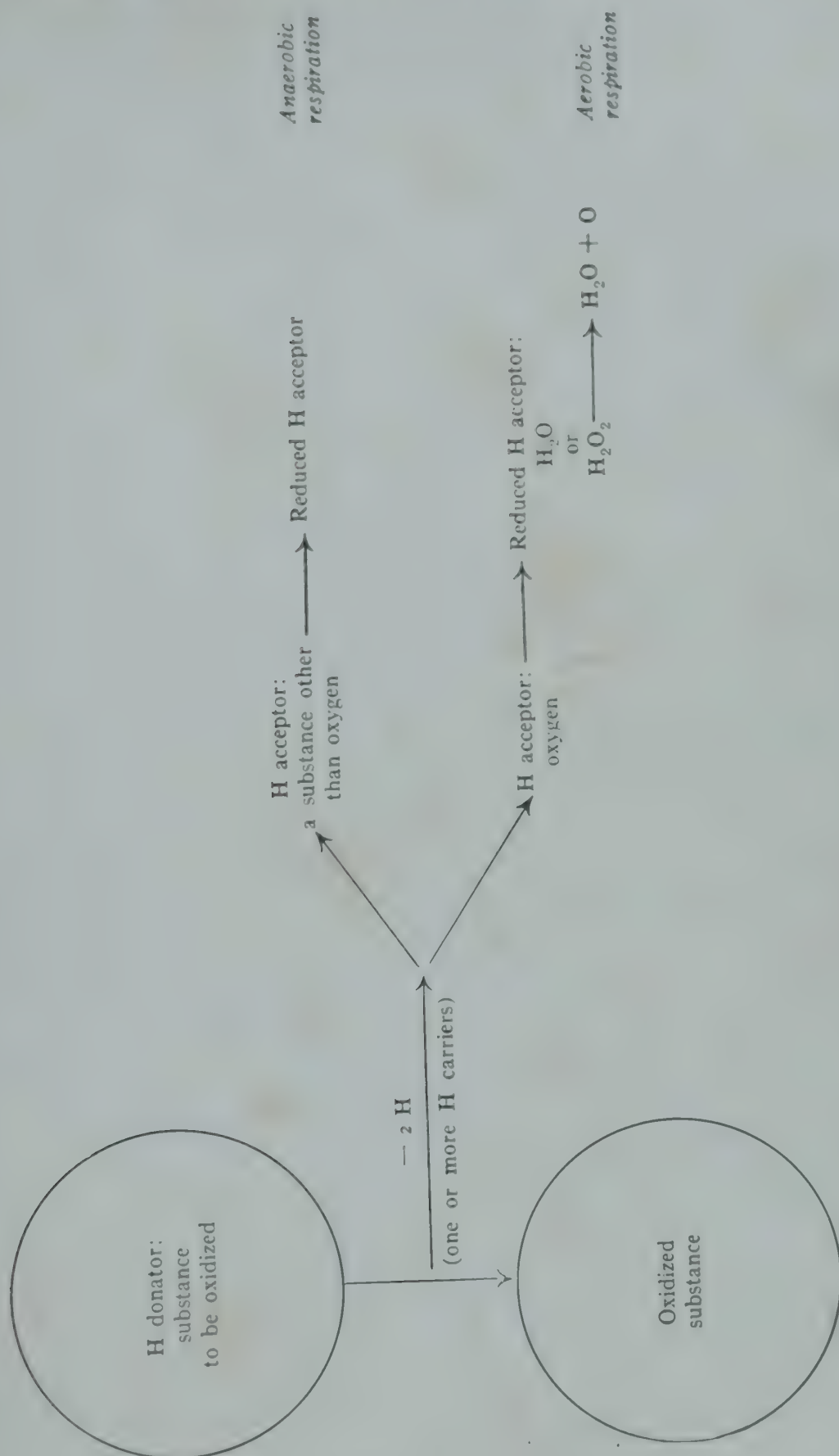
Obligate anaerobes do not contain catalase and the theory has been offered that the toxicity of oxygen for anaerobes is due to this deficiency and the accumulation of hydrogen peroxide. However, there is no evidence that hydrogen peroxide is formed by anaerobes when they are exposed to the air and consequently this explanation is not entirely acceptable. (Another enzyme that decomposes hydrogen peroxide and is active in aerobic respiration is **peroxidase**, which activates the oxygen of hydrogen peroxide to combine with an oxidizable substance instead of liberating free oxygen as catalase does.)

It has been pointed out that the hydrogen acceptors of obligate anaerobes and of facultative anaerobes living away from air are reducible substances other than oxygen. When such a substance accepts hydrogen it is reduced, and laboratory tests (known as reduction or reductase tests) may be performed to determine the change from the oxidized to the reduced form of a substance which is acting as a hydrogen acceptor in a culture, as, for example, in the reduction of nitrates ( $-NO_3$ ) to nitrites ( $-NO_2$ ), the change from oxidized methylene blue to the reduced, colorless or leuco-methylene blue, and the fading of litmus milk due to the shift from the oxidized to the reduced form of the indicator.

Table 7 presents a scheme comparing a biological oxidation in aerobic and anaerobic respiration.

**Products of Glucose Dissimilation.** Each step in the breakdown of glucose produces different substances and the nature of these products will depend on the bacterium fermenting the sugar (or rather on its enzymes) and the conditions under which it is growing, especially the amount of oxygen present. The more complete the decomposition of the sugar the greater is the amount of energy liberated to the cell. If a molecule of glucose is completely oxidized it will be broken down to carbon dioxide and water with maximum energy release. Usually the sugar is not decomposed completely, but instead acids, aldehydes, alcohols, carbon dioxide and sometimes hydrogen are the end products of fermentation. Further oxidation of the intermediate products resulting from incomplete oxidation is accompanied by the liberation of more energy to the cell. The breakdown of glucose is never accomplished by a single reaction; instead it is a step-wise process, a series of reactions, in which the sugar is gradually decomposed into smaller and smaller molecules. Consequently in any fermentation a number of products are present simultaneously. The following equations represent some





common oxidations of glucose ( $C_6H_{12}O_6$ ) in which the amount of energy released is expressed in terms of the large calorie,\* a measurement of heat energy:

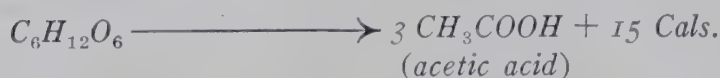
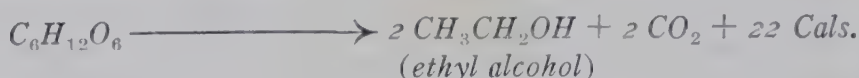
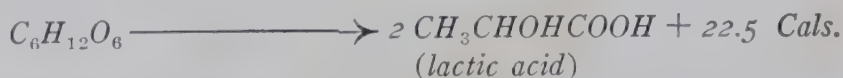
Complete aerobic oxidation:



Partial aerobic oxidation:



Anaerobic oxidations:



### Fermentations in the Identification and Classification of Bacteria

Under standard environmental conditions each bacterium ferments a given carbohydrate in a characteristic fashion with certain intermediate and end products. The kinds of products resulting from fermentations, therefore, may help in identifying certain species. For example, two very similar organisms *Aerobacter aerogenes* and *Escherichia coli*, may be distinguished by the fact that the former produces acetyl-methyl carbinol from glucose and the latter does not. More commonly pure cultures are grown in fermentation tubes containing carbohydrate indicator broths and these are examined after incubation for evidence of acid and gas formation. The three usual findings are: (1) no change in the indicator, no gas; (2) acid, no gas; (3) acid and gas. If an organism grew but produced no apparent change in a tube of, let us say, lactose broth, it means usually that the organism does not have the proper enzyme to attack that sugar. A change in the color of the indicator to that of a lower pH is evidence that the organism has the proper enzymes to digest and dissimilate the sugar with the accumulation of acid in the medium. The formation of acid and gas indicates still more complete oxidation including the breakdown of acids with liberation of gases, carbon dioxide or a mixture of carbon dioxide and hydrogen.

Bacteria are often grouped and even named according to the predominant product resulting from their fermentations. To illustrate one need only mention the lactic acid bacteria (members of the genera *Lactobacillus* and *Streptococcus*), the acetic acid bacteria (of the genus *Acetobacter*), the propionic acid bacteria (of the genus *Propionibacterium*) and the butyric acid bacteria (including many species of *Clostridium*). Many such products of carbohydrate dissimilation have commercial value, and in some instances the bacteria manufacturing them have been put to work on large-scale production in industrial fermentations.

\* A large calorie, or kilocalorie, equals the amount of heat required to raise one kilogram of water one degree centigrade.



# 15

## PROTEIN METABOLISM

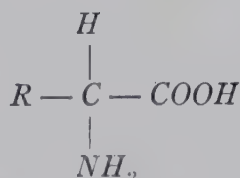
**Digestion of Proteins.** Just as complex carbohydrates must first be decomposed to simple sugars before they can be utilized, so proteins must be broken down to amino acids before they are available to bacterial cells. Amino acids are the units of structure or "building stones" which are linked together to form proteins, the largest and most complex of all molecules. The huge insoluble protein molecules are decomposed by extracellular digestive enzymes (**proteinases**) into molecules of decreasing size by a series of hydrolyses. The addition of water splits the protein into smaller molecules, **proteoses**, which in turn are hydrolyzed into still smaller molecules, the soluble **peptones**. The dissolution of a solid protein to the liquid state indicates that protein digestion or proteolysis has proceeded at least as far as the peptone stage, and consequently the liquefaction of protein is known as **peptonization**. (Old, spoiled milk becomes clear and watery when the milk protein, casein, has been peptonized.) Peptone molecules, however, are too large to diffuse through the cell membrane, and if the bacteria are to obtain any of this food, further extracellular decomposition is necessary. Peptones must be hydrolyzed to **polypeptides**, and polypeptides broken down into their component **amino acids**. At this stage protein digestion is complete; amino acids can enter the bacterial cells where they are further decomposed and incorporated into the cell substance.

The bacterial enzymes responsible for protein disintegration are, in general, most efficient in a slightly alkaline medium, and proteolysis does not accompany acid fermentation. The term **putrefaction** is applied to the decomposition of proteins since it is associated, especially beyond the amino acid stage, with the production of foul odors. Bacteria can be characterized often as either putrefactive (proteolytic) or fermentative (saccharolytic) types, for a single species is rarely capable of both protein digestion and active fermentation of carbohydrates. Proteolytic enzymes are specific in that separate enzymes act on proteins and on their simpler degradation products. The enzymes that hydrolyze proteins to proteoses, peptones and polypeptides are termed **proteinases**, whereas those that attack the polypeptides, splitting them to simpler molecules such as dipeptides and finally to amino acids, are known as **peptidasés**.

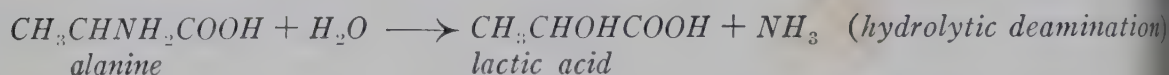
**Utilization of Amino Acids.** The amino acids supply the bacterial cells with nitrogen and other elements essential to the synthesis of protoplasm, and

they may also serve as a source of energy. Information on the processes involved in the utilization of amino acids is fragmentary and has been deduced mainly from the kinds of products which result when a pure culture is grown in media containing proteins or in synthetic media containing pure amino acids as the sole source of nitrogen. Ammonia and weak organic acids are the most common products of amino acid dissimilation although a variety of other substances are also formed.

Most of the biologically important amino acids may be represented by the formula



wherein  $-NH_2$  is the **amine** group,  $-COOH$  is the **carboxyl** group and R may be replaced in one amino acid by a single  $-CH_3$  group and in others by a radical containing a number of carbon and hydrogen atoms arranged in an open chain or in complex ring structures. The liberation of ammonia ( $NH_3$ ) or **deamination** may be accomplished in several ways, three of which are illustrated below in reactions involving the amino acid alanine:



Deamination is due to the action of endoenzymes, **deaminases**, and is commonly accompanied by the formation of organic acids such as the lactic, pyruvic and propionic acids shown in the above reactions. The kinds of products resulting from deamination depend on the amino acid attacked and the chemical process by which the enzymes effect deamination (as by hydrolysis, oxidation, reduction, etc.). Almost all bacteria can bring about the deamination of amino acids. Ammonia is a source of nitrogen to practically all bacteria whether they produce it by the breakdown of amino acids or are supplied it in the form of inorganic ammonium salts. Many bacteria will grow in a medium in which an ammonium salt is the sole source of nitrogen.

Another, not uncommon, method by which bacteria decompose certain amino acids is **decarboxylation** or the removal of carbon dioxide from the carboxyl group. A specific endoenzyme known as a **decarboxylase** is active in each decarboxylation, which results in the conversion of the amino acid to the corresponding **amine** and carbon dioxide. Thus the decarboxylation of the amino

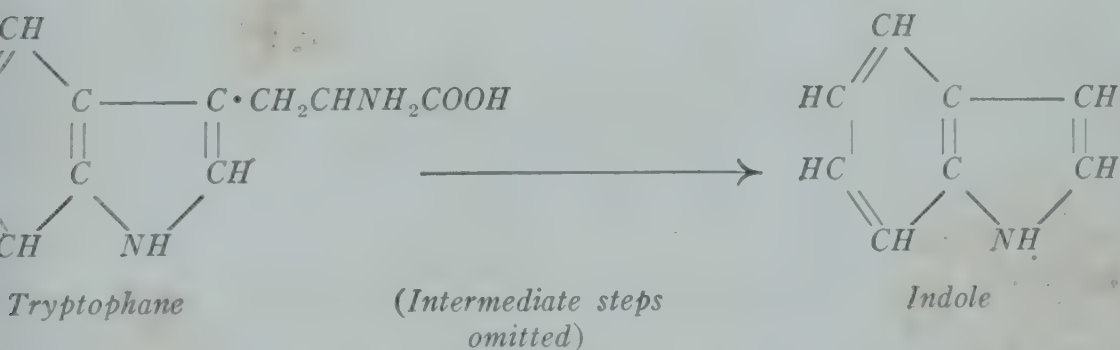


lysine results in the formation of the amine cadaverine and of carbon dioxide:



By combined decarboxylation and deamination some cultures may produce a variety of other substances. The liberation of ammonia from amino acids is encouraged by a slightly alkaline reaction (*pH* 7.5 to 8.0) and a slightly acid reaction (*pH* 5 or less) promotes decarboxylation and the production of amines. Amines in turn may be oxidized by some bacteria to form ammonia and other products. Certain supposedly poisonous amines known as **ptomaines** were once thought to be commonly responsible for outbreaks of acute gastroenteritis. Today it is believed that "ptomaine poisoning" rarely, if ever, occurs and food poisonings are due to other causes.

Deamination and decarboxylation are not the only ways by which bacteria dispose of amino acids and several kinds of reactions may proceed simultaneously. Consequently in a medium providing a number of different amino acids (whether it be meat, fish or another protein food in the home or a nutrient medium in the laboratory) a variety of breakdown products accumulates in the medium. Some bacteria decompose the sulfur-containing amino acids with the production of odoriferous compounds including hydrogen sulfide ( $\text{H}_2\text{S}$ ). Certain bacteria characteristically split the complex amino acid tryptophane with the production of indole:



Observing the action of an unknown bacterium on proteins and determining the products formed as a result of this action may contribute valuable clues in establishing its identity.

## DECOMPOSITION OF FATS

Certain bacteria are active in decomposing fats and oils. Such substances are not available as food to all bacteria, for many species are not equipped with the necessary lipolytic enzymes to digest them and lipids are often too low in water content to support bacterial growth. Bacteria that can attack fats may do so by hydrolyzing them to glycerol and fatty acids. The glycerol is an available energy source for many species, but fatty acids are not popular bacterial foods. A taste

of rancid butter is eloquent evidence that lipolytic microorganisms do exist and that butyric acid is one of the products of fat decomposition.

### PIGMENTS AND TOXINS, BY-PRODUCTS OF BACTERIAL METABOLISM

**Pigments.** Like the yeasts and molds, some bacteria produce a variety of pigments differing as to color and solubility and occurring inside or outside the cells. Many chromogenic bacteria retain a water-insoluble pigment inside the cells and as a result their colonies appear colored while the medium remains unchanged. Such is the red pigment of *Rhodococcus*, certain actinomycetes and the purple sulfur bacteria, the orange of *Staphylococcus aureus*, the yellow of *Sarcina lutea*, the violet of *Chromobacterium violaceum*, and the brown or black of *Bacillus mesentericus*. Most of the colored colonies which develop on air, soil and water plates contain intracellular, nondiffusible pigments. Other bacteria excrete pigments which may or may not be soluble in water. *Serratia marcescens* is a favorite in laboratory experiments because of its deep red color and a star performer in miracles involving the appearance of "blood" on bread and wood, is an example of a bacterium which produces an extracellular, water-insoluble pigment, **prodigiosin**. In cultures of *Pseudomonas aeruginosa* the medium as well as the colonies are colored bluish green due to a mixture of two water-soluble compounds, one a blue pigment, **pyocyanin**, and the other a green fluorescent pigment.

Chemical studies of bacterial pigments indicate that many of them are identical with, or closely related to, those occurring in vegetables and flowers. With the exception of the green and purple sulfur bacteria and a few others, there is little or no evidence that bacterial pigments serve any important function in the cell; instead they seem to be waste products of metabolism. Bacteriochlorophyll and a variety of reddish carotenoid compounds comprise the pigment system in the purple sulfur bacteria. Bacteriochlorophyll closely resembles, but is not identical with, the chlorophyll of green plants. Chromogenesis in general is greatly influenced by the composition and the pH of the medium, the incubation temperature, the intensity of light and the oxygen tension to which the cells are subjected. Even a superficial examination of *Serratia marcescens* cultures grown under different environmental circumstances will illustrate some of these relationships. In pour plates the surface colonies of *Serratia* may be deeply pigmented, while subsurface colonies are pale or colorless. If a culture of this same organism is held at 37° C and one at cooler, room temperature, pigmentation will develop more promptly and to a greater degree at the lower temperature.

**Toxins.** The growth of certain bacteria whether it be in a laboratory culture (*in vitro*) or in the living body (*in vivo*) is accompanied by the formation of **toxins**, substances poisonous to man and other animals. Toxins are products of cell metabolism just as enzymes, pigments, and waste products in general are the result of chemical activity associated with maintaining the life of the



There are two kinds of bacterial toxins: **exotoxins** and **endotoxins**. **Exotoxins** are soluble toxins which diffuse out of the living bacteria into the environment (the culture medium or the living host), while **endotoxins** are released inside the bacteria until freed on disruption of the cells. The nature of exotoxins and their significance in the production of disease will be discussed in the next chapter. The object of introducing them now is to point out that in the process of building up and breaking down cell substance (*i.e.*, metabolism) bacteria manufacture a wide variety of products; incidentally, some of these products are poisonous to man.

# 16

## ACTIVITIES OF BACTERIA IN NATURE AND INDUSTRY

The same biochemical reactions observed in the laboratory study of microorganisms are performed in nature by the yeasts, molds and bacteria. When milk sours, bacteria have fermented the lactose in the milk with acid production. When meat spoils it becomes soft and has an unpleasant odor; the enzyme-producing proteolytic bacteria are at work hydrolyzing the proteins of the meat to so-called peptones and further splitting the peptones to amino acids which finally are dissimilated into ammonia, organic acids, carbon dioxide, amines, hydrogen sulfide and other putrefactive products.

Food spoilage is in general a matter of the microbic fermentation and putrefaction of dead plant and animal bodies or of plant and animal products. In nature, microorganisms cause the decay of dead organisms by decomposing the complex molecules of body substance and transforming them into the mineral salts of the soil and the gases of the air. "Dust to dust" has real meaning. The chemicals once a part of living bodies are returned to the soil, and the soil is enriched and supports the growth of more green plants. Animals eat plants or other animals which have eaten plants, and thus the food cycle in nature is made complete.

### THE NITROGEN CYCLE AND SOIL FERTILITY

The fate of a nitrogen atom from its origin as part of a nitrate salt in the soil, through the bodies of one or more living organisms and its return to the soil in the form of a mineral salt is traced in the **nitrogen cycle**. Thus an atom of nitrogen in a nitrate compound may become part of the protoplasm of a green plant. If the plant is eaten by an animal the same atom may later be incorporated in an organic molecule of animal protoplasm, or it may be eliminated in the animal's excreta. In any event the plant and animal die eventually, and regardless of how many organisms have used the nitrogen atom, it will finally be mineralized in the soil and made available to another plant by the activity of microorganisms. The relationships of the various processes operating in the nitrogen cycle are illustrated in the accompanying diagram (Fig. 115) and described below. In this same way a molecule of any element in living organisms, such as carbon, sulfur or phosphorus, may be used over and over again, and



being part of the organic world, now one of the earth's mineral salts or gases in the atmosphere; and the course of their transformations may be described as the carbon, sulfur or phosphorus cycle respectively.

A fertile soil is a soil well supplied with the materials necessary for abundant growth. Green plants require carbon dioxide from the air and water from the soil.

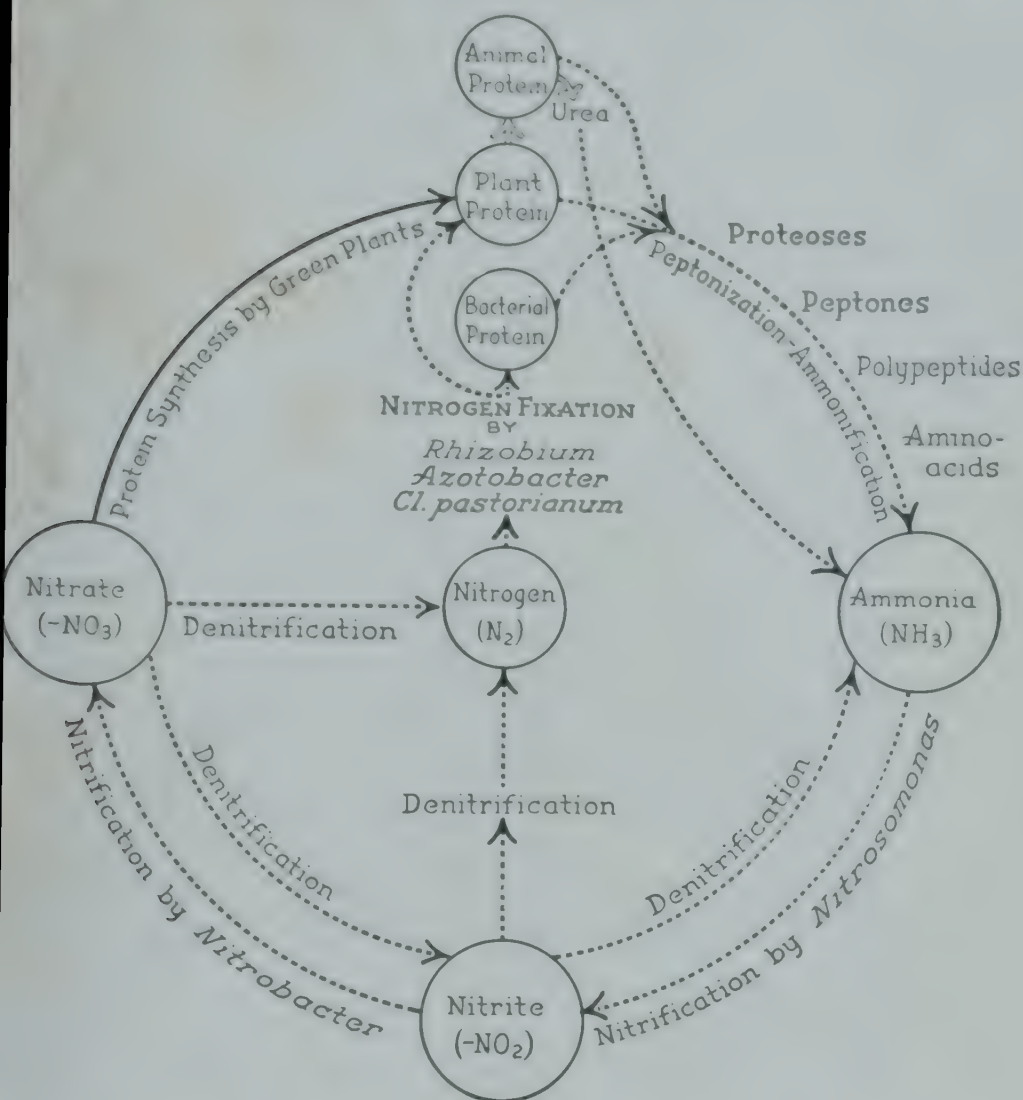


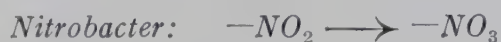
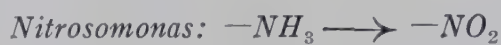
Fig. 115. Diagram of the nitrogen cycle. The dotted lines represent activities of bacteria and certain other microorganisms. (Adapted from Hilliard: *Textbook of Bacteriology and Its Applications*, Ginn & Co.)

Soil for the photosynthetic manufacture of sugar. Nitrates, phosphates, and other salts must also be provided by the soil for the synthesis of proteins of plant protoplasm. Since practically all cultivated green plants take up nitrogen in the form of nitrate ( $-NO_3$ ) salts, to be fertile a soil must be rich in nitrates, and any process which increases or decreases its nitrate content either enhances or diminishes its fertility.

**Peptonization and Ammonification.** Most crops withdraw considerable amounts of nitrates and other essential salts from the soil, and the farmer

restores this loss by applying fertilizers such as manure, compost, beef bone meal or nitrates to the land. But none of these fertilizers nor the of plants and animals that have died in or on the soil adequately serve the plants as food until first acted on by microorganisms. A great variety of prophytic bacteria are proteolytic; those in the soil decompose plant and animal proteins to peptones (**peptonization**) and then to amino acids. Probably all bacteria can liberate ammonia from amino acids by deamination, and in fertilized aerated soils this process of **ammonification** predominates over the methods of amino acid dissimilation. In the soil the free ammonia combines with sulfate, phosphate, chloride and other radicals to form the corresponding ammonium salts.

**Nitrification.** The important steps of changing ammonium salts to nitrates is the work of certain soil bacteria, nonsporulating bacilli of the genus *Nitrosomonas* and the genus *Nitrobacter*. *Nitrosomonas* organisms oxidize ammonia to nitrites, substances which are useless to the great majority of plants but necessary in the respiration of the *Nitrobacter*, a process by which nitrites are oxidized to nitrates. Thus the production of nitrates from ammonia or the process of **nitrification** is completed:



Both these groups of nitrifying bacteria are strictly autotrophic in that they are obliged to obtain energy from the oxidation of these nitrogen compounds in the same way that most other organisms use carbohydrates. Furthermore, they are obligate aerobes and can function only in well aerated soils. The practice of loosening the soil does more than ventilate plant roots; it also promotes nitrification and increases soil fertility. The importance of the nitrifying bacteria can be appreciated only when one realizes that no other organisms can substitute for their performance in the production of nitrates from ammonia. Although there are few species of nitrifying bacteria, they are widely distributed, being present in practically all soils.

**Denitrification.** Most of the soil's nitrate supply is the result of the putrefaction of nitrogenous organic matter with subsequent ammonification and nitrification as described above. If any interruption halts this sequence of events, soil fertility diminishes and crops suffer. Curtailment of nitrate production threatens whenever the oxygen supply in the soil is cut off as it is in water-soaked land. Then not only will the nitrifying bacteria cease to function, but also other bacteria growing **anaerobically** will destroy the nitrates already present, reducing them to nitrites and ammonia and liberating free nitrogen. Many kinds of bacteria using nitrates as hydrogen acceptors are able to reduce them to nitrites and thus undo the good work of the nitrifiers. Fewer bacteria can produce ammonia and nitrogen from nitrites, but these reactions also occur.



**Nitrogen Fixation.** The nitrogen gas resulting from denitrification escapes to atmosphere which is already four-fifths nitrogen and where it is useless to most organisms. Three kinds of soil bacteria and certain blue-green algae are exceptions, for these organisms can utilize elementary nitrogen in the synthesis of their protoplasm. The transformation of gaseous nitrogen into volatile, nitrogenous compounds is termed nitrogen fixation. The nitrogen-fixing bacteria belong to three different genera whose distinguishing characteristics are outlined as follows:

**Aerobic, nonsporulating bacilli**

- |                                                                  |                                          |
|------------------------------------------------------------------|------------------------------------------|
| (1) Free-living in the soil .....                                | <i>Azotobacter</i><br>(several species)  |
| (2) Living symbiotically in the roots of leguminous plants ..... | <i>Rhizobium</i><br>(one or two species) |

**Anaerobic, sporulating bacillus**

- |                                   |                                |
|-----------------------------------|--------------------------------|
| (1) Free-living in the soil ..... | <i>Clostridium pastorianum</i> |
|-----------------------------------|--------------------------------|



Fig. 116. Roots of a leguminous plant (scarlet runner bean) showing nodules which contain symbiotic nitrogen-fixing bacteria (*Rhizobium*). (From Holman and Robbins: *Book of General Botany*, John Wiley & Sons, Inc.)

Each of these organisms can grow, *i.e.*, synthesize proteins, in a medium containing a carbohydrate, a variety of mineral salts, but no nitrogenous compound, gaseous nitrogen being supplied by the atmosphere. The exact mechanism of nitrogen fixation is not known. Using energy obtained from the oxidation of the carbohydrate, the cells probably first fix  $N_2$  as ammonia ( $NH_3$ ) which is then combined with organic acids to form amino acids, the building blocks of proteins. In this way nitrogen may be fixed in the soil by the free-living *Azotobacter* and *Clostridium pastorianum* as it is in the roots of leguminous plants by the *Rhizobium*.



Fig. 117. Barred and branched forms of *Rhizobium leguminosarum*, types of cells found particularly in nodules of alfalfa and sweet clover. (From Henrici: *The Biology of Bacteria*, D. C. Heath & Co.)

In the case of symbiotic nitrogen fixation, the *Rhizobia* infect the young tender roots of plants which bear their seeds in a certain type of pod (legumes). Clover, alfalfa, soy beans and vetch belong to this category. In the roots of the growing plant the bacteria multiply and the plant's reaction is the formation of tumor-like nodules (Fig. 116). Finally a balance is struck between the bacteria and the plant when the association develops into one of mutual benefit or symbiosis. The plant furnishes the *Rhizobium* with carbohydrates, and the presence of the nitrogen-fixing bacteria increases the plant's supply of available nitrogenous compounds. The value of planting leguminous crops on land whose nitrogen content has been diminished by intensive cultivation is now evident. The roots of mature clover, alfalfa and the like are rich in fixed nitrogen, awaiting only microbic decay, ammonification and nitrification to replenish the soil with a good supply of nitrates. The practice of crop rotation is based on the facts that certain crops take more nitrogen from the soil than others, and that a crop of



luminous plants increases its fertility. Fortunately nitrogen-fixing bacteria are old wide in their distribution. To assure maximum symbiotic fixation, however, farmer can purchase seeds of leguminous plants which have been inoculated in cultures of *Rhizobium*.

Very little gaseous nitrogen is made available to the living world by natural agencies other than the nitrogen-fixing bacteria, although a small amount is fixed lightning during electrical storms.

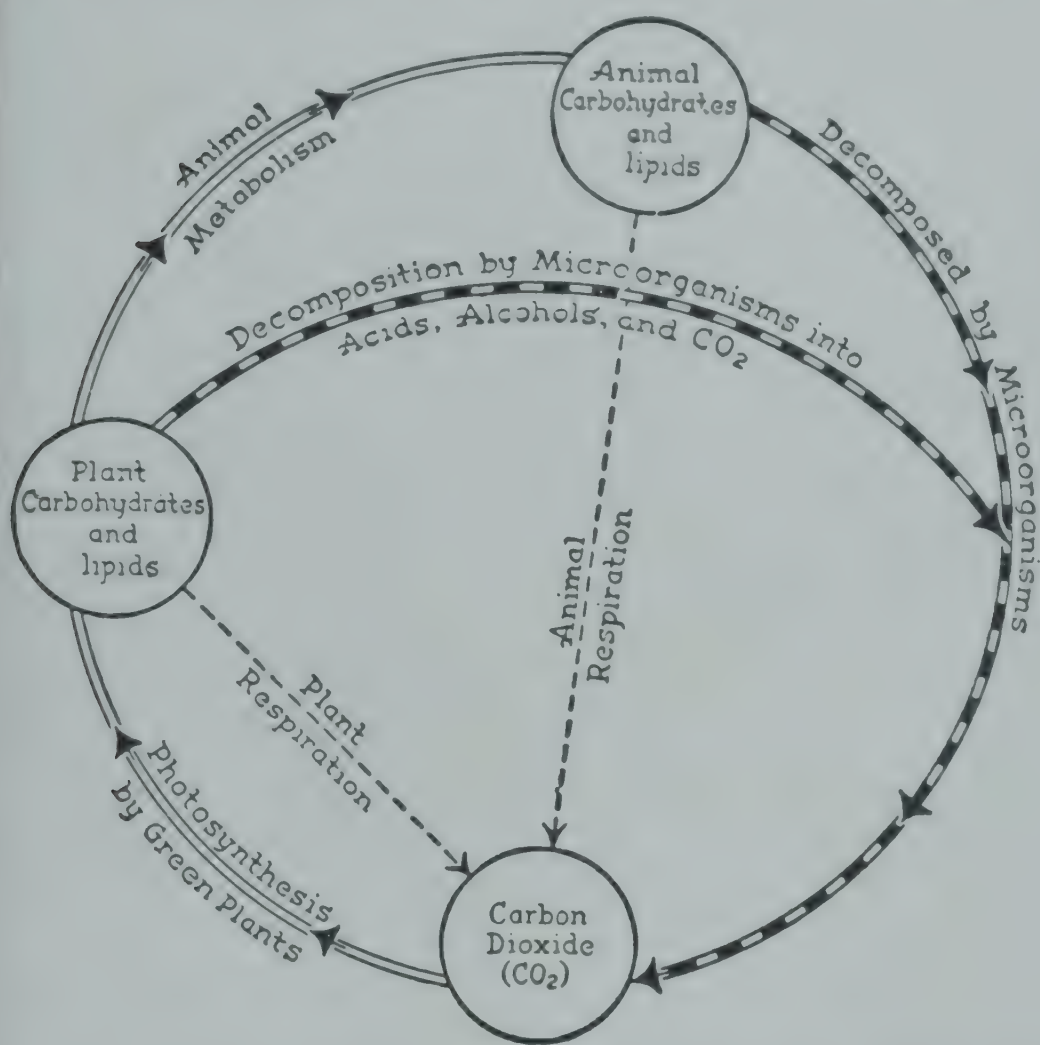


Fig. 118. Diagram of the carbon cycle. The barred lines represent activities of bacteria and other microorganisms. (Adapted from Hilliard: *Textbook of Bacteriology Its Applications*, Ginn & Co.)

### THE CARBON CYCLE

The carbon cycle in nature, including the role played by microorganisms, is represented diagrammatically in Fig. 118. By photosynthesis green plants combine the carbon dioxide of the air with water from the soil to form sugar which is later built into more complex carbohydrates as well as into plant lipids and proteins. Animals eat plants and from them make their own particular brands of carbohydrates and fats. The supply of atmospheric carbon dioxide, which

would soon be depleted if it were not continuously replaced, is maintained by the respiratory processes of plants and animals. Microbic decomposition of organic matter, either by fermentation or putrefaction, also results in the freeing of carbon dioxide into the air. The burning of wood, coal, leaves or any organic material is another form of decomposition that contributes this gas to the atmosphere.

### BACTERIA IN INDUSTRY

**Natural and Commercial Fermentations.** The important part played by bacteria in commercial fermentations, in the preparation of foods, in the manufacture of leather, linen, hemp, synthetic rubber and many other essential commodities can only be mentioned here. Bacteria are put to work making commercial solvents, such as ethyl alcohol and acetone as well as other alcohols and acids of industrial value. Butyl alcohol, amyl alcohol, isopropyl alcohol and butyric, oxalic, lactic, tartaric and acetic acids are produced by large-scale bacterial fermentations of cheap carbohydrates. Some of these acids may be converted into salts, notably lactates and citrates, which are used in drugs. In another commercial process bacteria produce butylene glycol which is a precursor of butadiene, a substance used in the manufacture of synthetic rubber.

Bacterial enzymes have been active at some stage in the production of many of our foods, coffee, cocoa, butter, cheese, sauerkraut and vinegar to name a few. The fermentation of sugars with the formation of large amounts of lactic acid is the property of certain bacteria (several species of lactobacilli and streptococci) that suit them for the task of producing the acid curd cheeses, sauerkraut and ensilage, the fermented green fodder relished by cattle. *Streptococcus lactis* growing in milk or cream produces the soft curd of cottage and cream cheese. The hard cheeses are made by allowing milk to undergo the same type of souring and then adding rennet, an enzyme obtained from the calf's stomach, to form the firmer curd. During a ripening process some cheeses gain their distinctive flavor, aroma and texture by supporting the growth of putrefactive and/or fermentative bacteria. Limburger, liederkranz, edam, cheddar and swiss cheeses are bacteria-ripened, just as camembert, gorgonzola and roquefort are mold-ripened cheeses. A mixture of streptococci (*Str. lactis*, *Str. cremoris*, *Leuconostoc citrovorum* and *L. paracitrovorum*) give butter its "buttery" flavor and are sold as butter starters.

The production of natural vinegar is the result of the fermentative action of yeast plus that of the acetic acid producing bacteria (*Acetobacter*). The sugars in fruit juices are first fermented by yeasts to form alcohol as in hard cider or wines, and then the *Acetobacter* organisms convert the alcohol to acetic acid. "Mother of vinegar," the slimy deposit seen in the vinegar keg, is a mass of acetic acid bacteria in their mucoid products.

Partial disintegration of plant and animal parts by naturally occurring bacteria is the basis of one step in the manufacture of leather and of the retting of flax and hemp. In the former process the hairs are removed from the hides



bacterial digestion, whereas in the latter, bacteria free the plant's strong basters by dissolving the pectin which binds them in the leaves and stems. The fibers of flax are manufactured into linen and those of hemp into rope.

**Microbic Assay of Vitamins.** Another application of microbiology is the use of yeasts and bacteria in measuring the vitamin content of tissues, foods, commercial vitamin preparations and other biological products. *I.e.*, in microbic methods of vitamin assay. Quantitative determinations of eight members of the vitamin B complex can be made by means of bacteria and four can be assayed by methods employing yeasts. The principles underlying these biological assay methods are briefly as follows:

(1) Certain yeasts and bacteria must be supplied with minute amounts of one or more vitamins (accessory growth factors) in order to promote metabolic activity and growth.

(2) In a liquid medium containing all the substances essential for metabolic activity and growth **except the vitamin to be assayed**, the test organism will show low or no turbidity and no acid production.

(3) With the addition of the vitamin to such a deficient medium, growth (indicated by turbidity) and metabolic activity (measured by acidity) are increased in direct proportion to the amount of vitamin added until an excess supports maximum development of the organisms.

In applying these principles to the method, the test organism is grown (a) in a series of tubes containing the deficient medium plus known and graded amounts of the vitamin (standard cultures) and (b) in a second series containing the same deficient medium plus known amounts of the test substance (vitamin content unknown). By comparing the degree of turbidity or the acidity of cultures containing the test substance with that of the standard cultures, the amount of the vitamin in the test substance can be determined. *Lactobacillus casei* and other lactobacilli have been the most useful of the bacteria in these microbic methods for the quantitative determination of such vitamins as riboflavin, pantothenic acid, biotin and folic acid.

**Antibiotics of Bacterial Origin.** Antagonistic substances of bacterial origin are described under the subject of microbial associations (Chapter 18). The production of certain of these antibiotics has today moved out of the research laboratory into the field of industry. Pharmaceutical houses have equipped plants for the large-scale manufacture of such antibiotics as tyrothricin, streptomycin and aureomycin as well as penicillin. Other antagonistic substances derived from bacteria give promise of clinical value. In the future these and newly discovered antibiotics may prove worthy of commercial production.

## Part 4

# Microbial Populations

## 17

### DEVELOPMENT OF BACTERIAL POPULATIONS—INHERITANCE AND VARIATION

#### METHODS OF REPRODUCTION

Bacteria are distinguished from other fungi by their method of reproduction. Transverse binary fission divides the coccus, bacillus or spirillum into two equal size daughter cells. Bodies which divide before fission and which represent bacterial nuclei have been demonstrated in a number of cells but a process similar to mitosis has not been observed. However, some provision for the proper distribution of hereditary traits between the two daughter cells is undoubtedly made. Methods of reproduction other than binary fission have been described and, in some instances, repeatedly observed. Although there is no obvious budding as in the yeasts, bacteria are known to undergo unequal fission under certain conditions. Lateral protuberances which resemble buds are sometimes seen in bacilli, particularly in old cultures or in cells growing under unfavorable conditions. It is possible that these cells are not reproducing, but are displaying abnormal morphology in response to the adverse environmental influences.

Another method claimed by some to be a reproductive process is the formation of **gonidia**. The protoplasm of certain bacteria is said to contract to form one to several granular bodies which are liberated when the cell wall disintegrates and each particle (**gonidium**) is supposed to be capable of germinating a bacterium. Furthermore, some bacteriologists assume that gonidia represent a filtrable stage in the life cycle of these bacteria. Experimental proof of the existence of gonidia as living reproductive bodies is lacking. Among the branching filamentous bacteria known as the Actinomycetes, **fragmentation** and **conidia** formation are common methods of reproduction. Each filament or hypha may break up into many short rods each of which can grow out into the long branching form. Many Actinomycetes develop a powdery surface growth which on microscopic examination is seen to be composed of chains of cylindrical, oval or spherical spores, the conidia. Conidia are formed by segmentation of the aerial hyphae followed by rounding up of each segment into a reproductive spore which is more resistant to drying than the vegetative cells. Under suitable conditions the conidia germinate into the branching filaments of the vegetative mycelium.



sexual reproduction, although often postulated, has not been demonstrated conclusively in any of the bacteria.

## GROWTH AND DECLINE OF A BACTERIAL POPULATION

**The Growth Curve.** If a few bacterial cells are introduced into a sterile liquid culture medium and the numbers of living cells are ascertained at regular intervals during a suitable incubation of the culture, several interesting and important facts are brought to light. The bacteria do not begin reproducing at

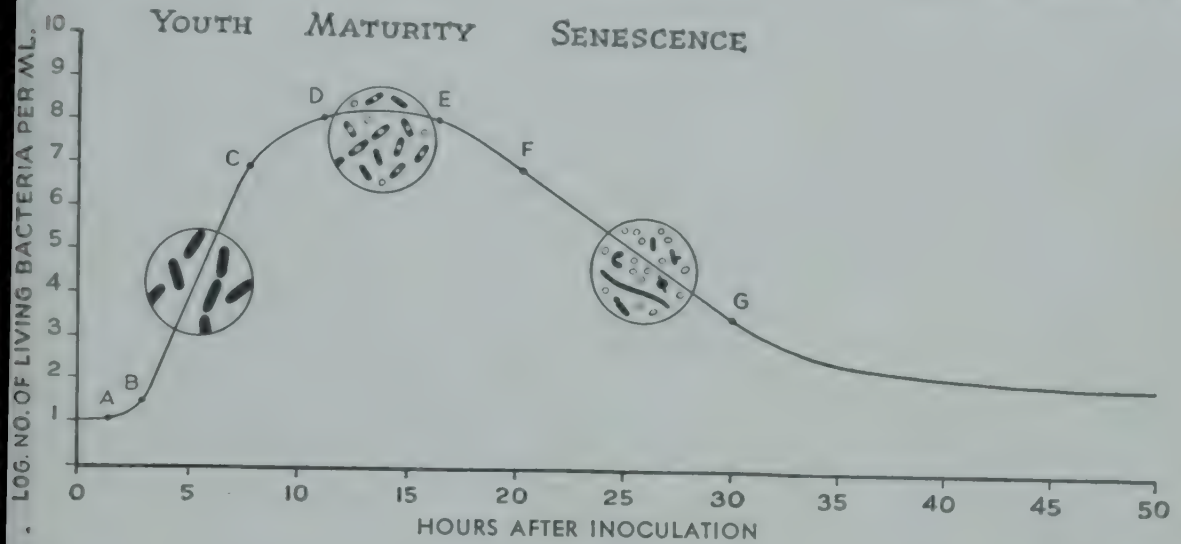


Fig. 110. Growth curve of bacteria showing morphological changes in a spore-forming bacillus from youth through maturity and senescence. 1-A, Lag phase; A-D, growth phase including (A-B) period of maximum multiplication rate, and (C-D) period of decreasing multiplication rate; D-E, stationary phase; E to end, death phase including (E-F) period of accelerating death rate, (F-G) period of maximum death rate, and (G to end) period of prolonged decline. (Adapted from Henrici and others.)

ence, but there is a period of adjustment to the new environment, a so-called **lag phase**, in which the original number of cells remains constant. The length of the lag phase depends on the species of bacteria and also largely on the age of the culture from which the inoculum was taken. If the culture is an old one several hours may elapse before the bacteria begin to reproduce in the new medium, while the lag phase may be reduced to practically *nil* if the inoculum is from a very young culture.

In any event, after an initial period in which there is no cell division, the numbers of bacteria begin to increase, slowly at first and then rapidly. These are, respectively, the times of increasing and maximum reproduction and together they comprise the **growth phase** of the culture. It is common for the rate of reproduction to reach a maximum at which each bacterium divides every 20 or 30 minutes, and for this period of maximum growth to last for several hours. By continuing to remove samples from the culture and determining the numbers of viable cells it is learned that there follows a slackening of the repro-

duction rate and the culture next enters a **stationary phase** in which there is no increase or decrease in the bacterial population.

Thus the growth phase of the culture is terminated and, for a short time, the rate of reproduction and the death rate are equal. Apparently the bacteria encounter circumstances such as the accumulation of their own waste products which inhibit cell growth and fission. Gradually the population decreases; now there are more bacteria dying than reproducing, and the culture is said to be in the **death phase**. After a period of maximum death rate, only a small proportion of the bacteria remain alive, and during a period of prolonged death their number slowly diminishes until relatively few living cells persist. The last cell may not die for days, weeks or even months, depending on the kind of bacterium in the culture. If the logarithms of the numbers of living cells are plotted against time, a **growth curve** is obtained which represents the rise and fall of the population in a pure culture (Fig. 119).

**Cellular Changes During Growth.** During the various phases of the culture's growth morphological and physiological changes in the cells are observed. At the start of the growth phase the bacteria attain their largest size; they stain deeply and evenly and they are highly susceptible to adverse influences such as the lethal action of heat and chemicals. As the culture matures the cell size diminishes, their affinity for ordinary bacterial stains lessens, deep-staining granules may appear and, in those species capable of spore formation, endospores develop. As the death phase advances, bizarre **involution forms** are common; rod-shaped bacteria tend to branch, bud or elongate into filaments, while cocci and vibrios as well as some bacilli may "blow up" into spherules. In an old culture pale-staining "ghost" forms are often evident, bacteria which were once gram-positive become gram-negative, many senile cells are vacuolated and the majority of spore-forming bacilli are in the free spore stage.

The hardy individuals that persist in old cultures are usually more difficult to kill than their ancestors were in the growth phase of the same culture. This change in resistance is especially marked in the sporulating bacilli, but it also obtains to a lesser degree in the case of the nonsporulating bacteria. Dead bacteria of certain species soon lose their identity and seem to melt away by a process of self-digestion or **autolysis**. Variations in shape, size and staining properties due to old age are more common in some species than in others. Moreover, the time necessary for the development of the growth phases and for the entire life span of a culture differs with different bacteria. Most of the bacterial cultures provided for the student's laboratory work reach maturity and are in the death phase in about 24 to 30 hours.

## BACTERIAL INHERITANCE

A study of the heredity of any organism reveals that offspring resemble their parents and yet no two individuals are exactly alike. The resemblance is generally great enough to make it plain that parents and offspring belong to the same



species, but no animal or plant is ever an exact replica of a parent. Such would have to be the case where sexual reproduction is involved, for in the offspring of sexual union there is a new combination of traits, some of which were contributed by the father and some by the mother. What is the situation in bacterial inheritance where there is only one parent and that parent gives up its life, so to speak, to become two daughter cells?

In general, bacterial inheritance seems to be like that of other organisms; the offspring usually resemble the parent, but variations also occur. In most one-celled organisms and in all higher plants and animals hereditary factors (genes) reside in a specialized nuclear constituent of the cells, namely the chromatin of the chromosomes. Cell division in these organisms is preceded by mitosis, a process of orderly change in the nucleus which insures equal distribution of the chromatin and, therefore, of heritable characters to the two new cells. What takes place in the mature bacterial cell before it divides? If not all the protoplasm but only certain material (analogous to chromatin) is responsible for bacterial inheritance, is there something like mitosis which arranges for its equal distribution before fission occurs?

The answers to these questions are unknown. Their solution may be nearer when more is known about the nature of the bacterial nucleus. It is difficult to believe that cell division in bacteria is a haphazard affair. Considering the frequency of bacterial fission (it is possible for a single cell to pass through 20 generations giving rise to over half a million organisms within a 10-hour period) and the fact that every cell division is a chance for variation, bacterial inheritance is remarkably constant. Cocci reproduce cocci, the offspring of staphylococci are staphylococci and the same is true of other morphological types of bacteria. Physiological traits such as biochemical reactions and pathogenicity as well as size, shape, cell grouping and other morphological characteristics usually appear to pass unchanged from generation to generation if the bacteria are subjected to the same growth conditions.

## VARIATION

In a pure culture, bacteria may occur which differ in one or more respects from the familiar parent cells. This variation may be due to a change in the environment or to what appears to be a spontaneous change in the heredity of the bacteria. In higher plants and animals the sudden appearance of a permanent variation is known as a **mutation** and is related to alterations in the hereditary material (genes) of those organisms. Generally bacterial variations are due to environmental changes and are only temporary modifications. Mutation-like variations in bacteria occur less frequently and, although they may not be permanent, they exhibit great stability.

Changes in the environment probably bring about variation in at least two ways: either the original culture contains the variant in small numbers and the new environment selectively encourages its growth, or the change in environment acts directly on the "normal" cells to cause the appearance of one or more new

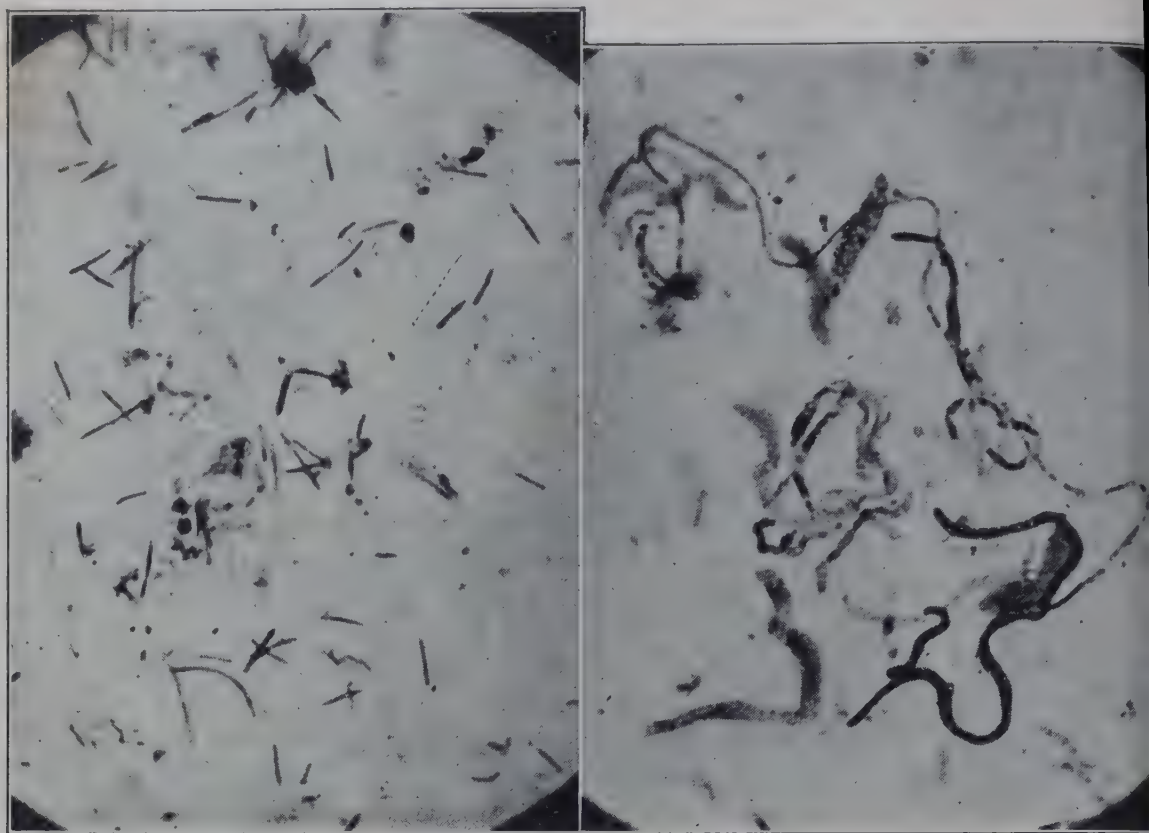


Fig. 120. Morphological variation of an anaerobic bacillus (*B. necrophorus*) growing on different culture media. Left, four-day culture grown on blood agar; right, four-day culture grown on blood agar to which ascorbic acid was added. Gram stain  $\times 1150$ . (Dack, Dragstedt, and Heinz: *J. Infect. Dis.* 60:341, 1937.)

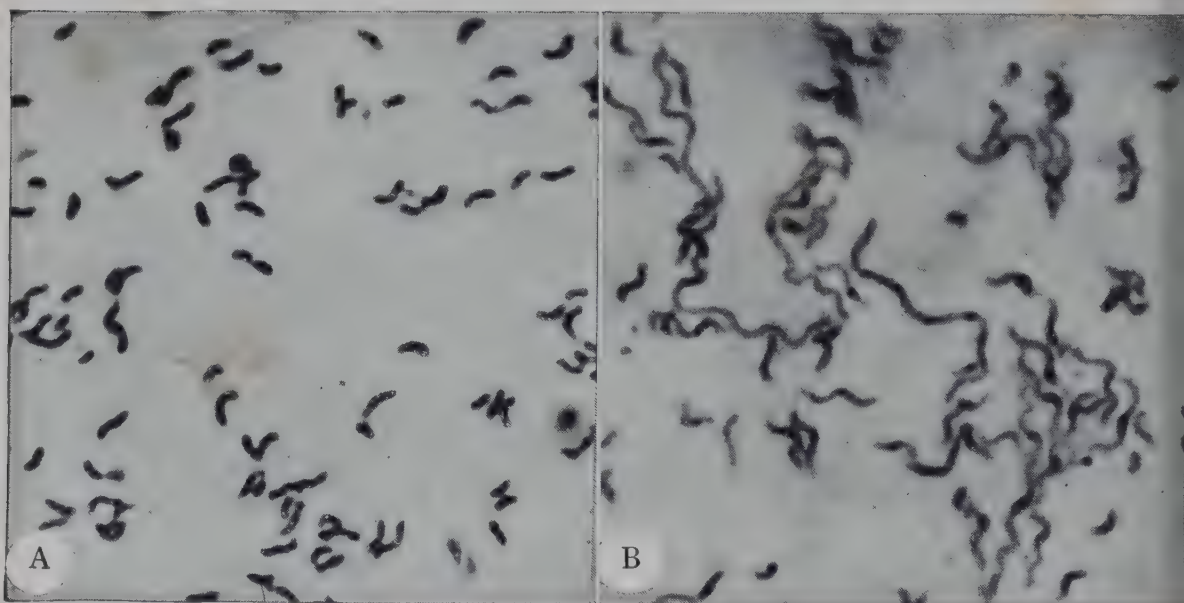


Fig. 121. Variation in cellular morphology of a vibrio due to aging of the culture. Left A, three-day culture; right B, six-day culture of the same organism growing on the same culture medium. (Kelly, F. C.: *J. Infect. Dis.* 74:93, 1944.)



characters. Gene mutations have been induced experimentally in the mold *Aspergillus crassa* by exposing the spores to ultraviolet light, x-rays and mustard gas, while many variants of *Penicillium notatum* have been produced in the laboratory by bombardment of the spores with neutrons. There is evidence that environmental factors may induce similar mutations in bacteria. An important contribution to this field was the demonstration that a chemical substance could induce the transformation of pneumococcal types. By growing a rough, nonencapsulated pneumococcus, which was derived from a smooth Type III culture, in the presence of the capsular substance or desoxyribonucleic acid isolated from the capsular substance of another type, such as Type III, a strain of encapsulated Type III pneumococcus is produced. Bacterial variants may appear spontaneously irrespective of the environment, and a recent review states: "In all thoroughly analyzed cases, we see that bacterial variation, including apparent hereditary adaptation, is the result of sudden spontaneous mutations."

The usual environmentally induced variation observed in bacteria is transmissible only so long as the environmental influence is operating. For example, it is common for morphological variants to appear in the death phase of an old culture, but if these degenerate bacteria are transferred to fresh culture media they will produce the familiar parent type of cell. If, however, the variant is grown under the continued influence of an unusual environmental factor the variation will persist, and the variant, being better adapted to the new conditions than the parent type cell, may predominate and even completely displace the "normal" type. In most instances as soon as the organism is returned to its former growth conditions it will revert to the character of its predecessor, although sometimes an established variation is maintained in subsequent generations even in the absence of the original environmental stimulus. When variations are permanent or at least stable through a great many generations, the variant is considered to be a new strain or type of the parent species. Thus there are avirulent strains of the diphtheria bacillus, sulfonamide-fast strains of pneumococci, asporogenous strains of the normally spore-bearing anthrax bacillus and many other examples of per-

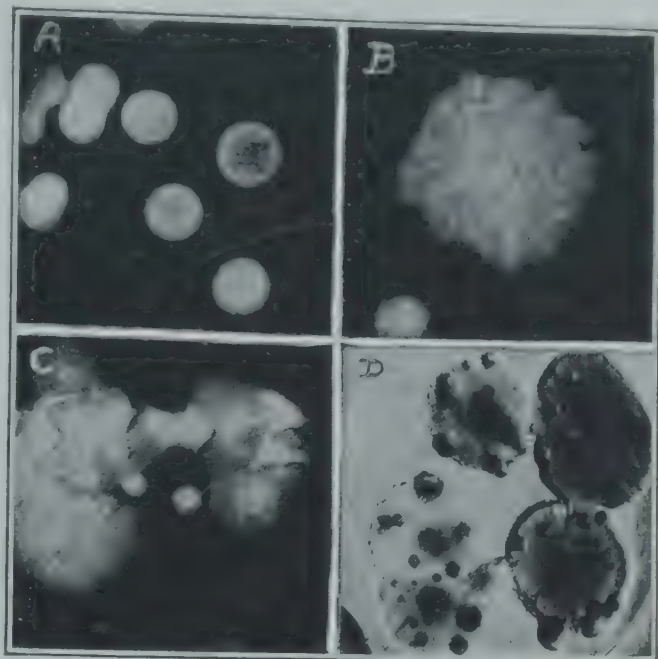


Fig. 122. Types of colonial variation. A. Smooth type colonies; B. rough type colony; C. mixture of smooth and rough colony variants; D, secondary, daughter colonies growing as papillae on parent colonies. (From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Appleton-Century-Crofts, Inc.)



Fig. 123 a



Fig. 123.b





Fig. 123 c

Fig. 123 a, b, c. Variant colonial forms of a micrococcus (*Micrococcus tetragenus*). (1) Mucoid yellow, (2) smooth yellow, (3) mucoid white, (4) smooth white, (5) translucent (the extra colony is a contaminant), (6) mucoid pink, (7) smooth pink, (8) rough pink, (9) mucoid-pink-yellow, (10) smooth pink-yellow, (11) rough pink-yellow, (12) mucoid brown, (13) smooth brown, (14) rough brown, (15) colony composed of bacillary forms. Sector formation may be noted in the smooth yellow (5), mucoid pink (6), and smooth (7) colonies. (From Reimann, H. A.: *J. Bact.*, 49:499, 1937. Reprinted from Dubos, R.: *The Bacterial Cell*, Harvard University Press.)

manent divergence in the morphological and physiological characters of species.

**S  $\rightarrow$  R Colony Variation.** One of the most frequently observed examples of bacterial variation is that of colony form. Variation in form, particularly that of colony morphology, has been referred to as bacterial **dissociation**, although this term is considered an unfortunate one since differentiation of bacterial species into types cannot be likened to chemical dissociation. Commonly a pure culture gives rise to moist, glossy, raised colonies (smooth or S type), but on continued cultivation there may appear flat, dull, rough-surfaced, sometimes wrinkled colonies (rough or R type). Colonies of the S type are creamy or butyrous in consistency, have even margins and appear homogeneous on microscopic examination; R type colonies are generally dry, at times even membranous or brittle, have irregular margins and appear granular under the microscope. In broth cultures, growth of the S type results in an even suspension of cells causing uniform turbidity throughout the medium. The R variant, on the other hand, gives rise in a liquid medium to a granular growth which soon settles out leaving the medium clear above a sediment. In physiological saline, cells of the R type clump spontaneously (self agglutination) and sink out of suspension, whereas the S type organisms remain evenly dispersed throughout the liquid.

The "normal" form of most bacteria is that of the smooth (S) type, and the common variation is S  $\rightarrow$  R, *i.e.*, the smooth parent type gives rise to rough variants. Organisms which are smooth type when first isolated often produce rough variants spontaneously on prolonged cultivation in the laboratory. The change from S  $\rightarrow$  R is a gradual one; at first only a few cells in the culture are the R type, but the proportion of rough to smooth forms may increase in subsequent generations until the R type predominates or appears to constitute the entire population of the culture. Laboratory techniques are known which induce or hasten the S  $\rightarrow$  R variation, and recent investigations describe methods that prevent the development of rough forms in certain cultures. Reversion of the rough back to the smooth type (R  $\rightarrow$  S) rarely occurs spontaneously.

Visible changes of the S  $\rightarrow$  R variations are accompanied and, in fact, are caused by fundamental changes in the bacterial cells. The cells in a smooth culture are grouped differently than those in a rough culture of the same species. The bacilli of the colon-typhoid-dysentery group, for example, occur singly and in pairs in the smooth form, but rough variants remain attached after division to form short chains. Encapsulated bacteria like pneumococci lose their capsules in the transformation from smooth to rough type. Flagellated motile bacteria have a tendency to become sluggish or nonmotile in the rough form, but this is not always the case. Of great practical significance is the decrease in disease-producing power that is associated with the S  $\rightarrow$  R variation in most pathogenic bacteria. With the exception of the anthrax bacillus, pathogens are more virulent in the smooth phase and undergo a lessening or complete loss of virulence with variation to the rough form. However, the essential factor in the transformation is a change in the chemical composition of the bacterial cells. With S  $\rightarrow$  R



variation there is a loss of substance from the surface of the "normal" (S) cells. Not only is this loss responsible for decrease in virulence, but it also causes a difference in the immunological (antigenic) properties of the bacteria.

The variations associated with the change from smooth to rough type may be summarized as follows:

#### SMOOTH (S) TYPE

- (1) Broth cultures uniformly turbid
- (2) Suspensions in saline (0.85 per cent NaCl) stable and remain cloudy
- (3) Flagellated species usually motile
- (4) Capsulated species show capsules
- (5) Surface substance (antigens) present
- (6) Pathogenic species generally virulent
- (7) Biochemically active
- (8) "Normal" morphology of cells

#### ROUGH (R) TYPE

- (1) Sediment in broth cultures; supernatant fluid clear
- (2) Suspensions in saline clump spontaneously and settle out
- (3) Motility reduced or absent
- (4) Capsules absent
- (5) Surface substance (antigens) lacking
- (6) Virulence greatly reduced or absent
- (7) Biochemical activity reduced
- (8) Tendency toward abnormal forms

**Other Colony Forms.** Besides rough and smooth there are other variations in colony form. Certain bacteria develop very moist, glistening, gummy or

**Mucoid (M type) colonies** in which the cells are heavily encapsulated. On subsequent cultivation the most frequent variation in a mucoid culture is from the M through the S to the smooth type colony. Another not uncommon variation in a pure culture is one of colony size. Minute, sometimes barely visible, dwarf colonies may appear among normal size colonies. In these there seems to be no difference in cell morphology from that of the parent type. In old plate cultures, small, secondary or daughter colonies may develop as knobs or papillae on the surface of the senescent original colonies. The cells in daughter colonies are bizarre in form and there is evidence that they also vary from the parent type in being able to

utilize as nutrients substances left unused by the bacteria of the original colony. Many believe that cells of the daughter colony are nourished by substances

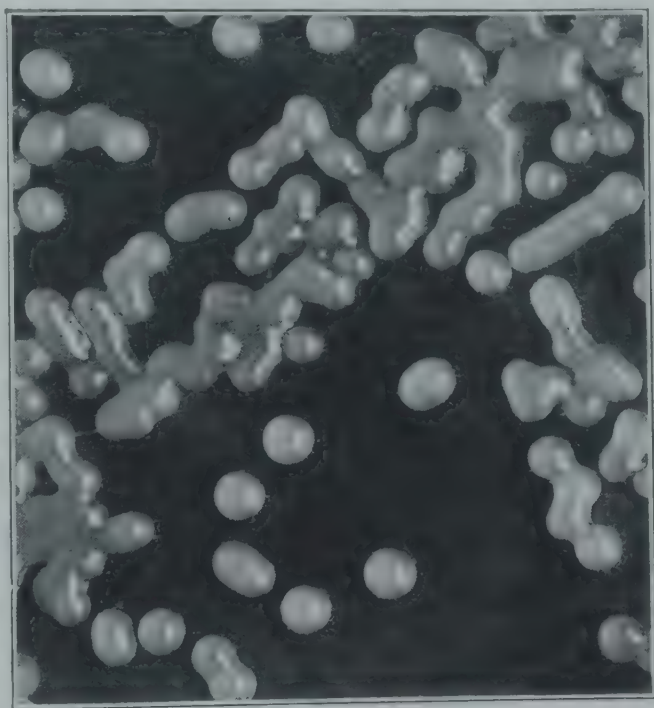


Fig. 124. Mucoid colonies of a streptococcus (*Leuconostoc mesenteroides*) isolated from sugar-cane juice growing on a raw sugar agar. (McCleskey, Faville and Barnett: *J. Bact.* 54:697, 1947.)

liberated from dead and disintegrating cells of the primary growth, a kind of cannibalism.

Variation in pigmentation is common, the variant usually showing a loss of color as compared to the parent type colony. Well isolated, aging colonies son

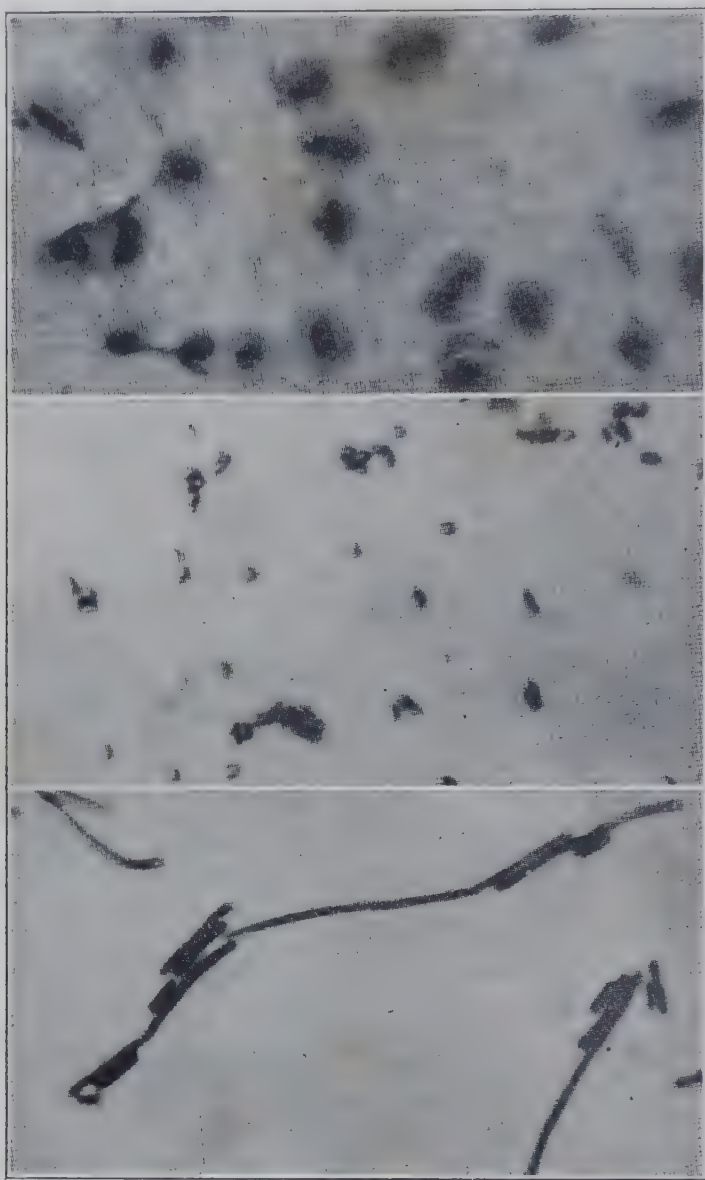


Fig. 125. Variant cell forms from mucoid (M), smooth (S) and rough (R) phase cultures of *Aerobacter aerogenes*. (Top) Encapsulated organisms from culture in the mucoid (M) phase.  $\times 1,200$ . Modified Hiss capsule stain. (Center) Nonencapsulated cells from culture in smooth (S) phase.  $\times 1,200$ . Gram stain. (Bottom) Filamentous cells from culture in rough (R) or transitional rough-smooth phase.  $\times 1,200$ . Gram stain. (From Osterman and Rettger: *J. Bact.* 42:699, 1941.)

times contain wedge-shaped portions or **sectors** which differ from the rest of the colony owing to the multiplication of variant cells in that region (Fig. 123). A pigmented colony may bear a white sector, a sporulating bacillus may form an asporogenous sector or texture and consistency may distinguish a sector from the rest of the colony.



**H  $\rightarrow$  O Variation.** German workers first noticed that flagellated, motile *typhoid* bacilli form a thin, flat, spreading growth while nonflagellated, nonmotile variants of the same bacteria produce discrete rounded colonies which show no tendency to spread. They named the flagellated form, which covered the surface of the agar like a film, the *Hauch* form (Ger., *Hauch*, breath or exhalation), and the nonflagellated variant, the *Ohne Hauch* form (Ger., *ohne*, without). Flagellated and nonflagellated variants of motile bacilli are now referred to as H and O forms respectively. H  $\rightarrow$  O variation is independent of S  $\rightarrow$  R variation since any combination of these characters may be found in variants of certain bacteria. Loss of flagella involves more than loss of motility; with loss of flagellar (H) substance there is a change in the chemical structure of the cell's surface which causes the H and O variants to react differently with matching immune serum. In the presence of such serum flagellated bacilli clump (agglutinate) rapidly, forming relatively large fluffy clumps (described as H agglutination), while the nonflagellated variants of the same organism slowly produce fine granular clumps (O agglutination). In the animal body the flagellar (H) substance and the substance of the bacterial cell body (somatic or O substance) stimulate the production of different kinds of antibodies. This fact has practical significance in the identification of certain motile pathogenic bacteria such as the typhoid and paratyphoid bacilli.

**Physiological Variation.** Pure cultures of bacteria may produce variants which differ in behavior as well as in appearance. Divergence between strains originating from the same culture are often expressed by variation in physiologic properties such as fermentation reactions, proteolytic power, nutritive requirements, action on red blood cells (hemolysis), production of pigment, indole, hydrogen sulfide, etc. As in the case of morphologic characters, some bacteria are more constant in their behavior traits than others, *i.e.*, there are stable and unstable strains. Only reactions which occur regularly are useful in the identification of a given organism. Fermentation reactions, for example, valuable in identifying most enteric bacilli, are practically worthless in the case of Friedlander's bacillus because there are so many strains of this organism which vary in their actions on carbohydrates.

If well established strains of a bacterium behave differently in only one or two respects these differences offer no confusion in determining the identity of the organism. There are, for instance, strains of the colon bacillus (*Escherichia coli*) which differ in their action on sucrose; the sucrose-fermenting strain is known as the *communior* variety (*E. coli* var. *communior*), whereas the non-sucrose-fermenting strain is *E. coli* or, in the older nomenclature, *B. coli communis*. Hemolytic and nonhemolytic varieties of *Staphylococcus aureus* and several other bacteria are commonly encountered. One of the first recognized variations in biochemical reactions was that of a colon bacillus the colonies of which did not ferment lactose and consequently did not change the color of the indicator on a lactose agar plate. On aging, however, these same white colonies developed papillae, daughter colonies, which did ferment lactose. Subcultures from

the daughter colonies were always lactose-fermenting, whereas cultures inoculated from the white portion of the original colony invariably gave rise to nonlactose-fermenting colonies which in turn eventually produced the lactose-fermenting variant. This ever-varying strain was named *B. coli mutabile*.

Variants of the same organism may differ from one another in nutritional requirements, *i.e.*, in the type of substrate they can decompose or synthesize into cell substance. One strain of the typhoid bacillus requires the amino acid tryptophane, while another does not. One strain of a bacterium may demand a trace of a certain growth factor or vitamin in the medium, while another strain of the same organism will grow without it. Sometimes bacteria can be "trained" to do without substances which were once essential to their growth. Thus on primary isolation from the body the whooping cough bacillus requires blood for its development, but after cultivation on media containing less and less blood it can be grown on ordinary nutrient agar.

Since the biochemical reactions brought about by bacteria are due to the actions of enzymes, physiological variation may be considered as a matter of change either in the kinds of enzymes formed by the cells or in the type of enzymes operating in a given environment. It is now well known that the constituents of the medium may influence the production of enzymes by bacteria. Most enzymes elaborated by a given organism are formed regardless of the composition of the medium in which it is growing; these have been termed the **constitutive enzymes**. Other enzymes, known as **adaptive enzymes**, are formed in detectable or appreciable amounts only if the medium contains, in each case, the specific substance which the enzyme attacks. One or a series of transfers through a medium containing the enzyme-stimulating substance may be necessary before enough of the enzyme is formed to utilize the substance. Although many physiological variations are undoubtedly adaptations induced by the environment, there is evidence that spontaneous mutations, independent of the substrate, may also be responsible.

**Variation in Virulence.** That a pathogenic bacterium may vary in its power to produce disease has already been mentioned in the discussion on  $S \rightarrow R$  variation. Except in the case of the anthrax bacillus, organisms from smooth colonies are more virulent than those from rough colonies, and mucoid colonies carry maximum virulence. Pathogens become less virulent the longer they are grown in culture media outside the body of a natural host, but their virulence can be regained and enhanced by repeated passage through a series of susceptible laboratory animals. If a pathogen of high virulence is passed from a susceptible animal to a series of more resistant animals its virulence becomes reduced or attenuated. Bacteria can be manipulated in other ways to attenuate or completely destroy their virulence. Pasteur, for instance, discovered that growing the anthrax bacillus at a temperature above its optimum causes loss of virulence. The power to produce disease and, therefore, variation of that power is dependent not on one but on several properties residing in most pathogens. There are avirulent strains of the diphtheria bacillus, tetanus bacillus and other bacteria



have lost the ability to produce toxins. Nonencapsulated strains of pneumococci are avirulent and are readily phagocytosed in the body. Other pathogens may lose one or more of the factors which make multiplication in or invasion of the host's body possible.

**Variation in Sensitivity to Drugs.** Different strains of the same organism frequently vary in their susceptibility to the action of chemotherapeutic agents. Long before the sulfonamide compounds were available, drug-sensitive and drug-resistant (drug-fast) strains of protozoa were known and arsenic-fast strains of syphilis spirochete had been encountered in arsenic-treated cases. With the introduction of each new sulfonamide drug more reports appeared describing sulfonamide-tolerant strains of various bacteria. Drug-fast strains are apt to develop in cases receiving inadequate doses of a drug. *In vitro* tests show that serial cultivation of a sensitive organism in laboratory media containing increasing amounts of a drug will usually produce a resistant strain. In other words, bacteria can be "trained" to grow in concentrations of a chemical agent which were once inhibitory. Sulfapyridine-fast strains of pneumococci have been developed in the laboratory and observed *in vivo*. Sulfadiazine administered to army personnel in small doses to prevent outbreaks of respiratory infections by hemolytic streptococci resulted in less effective prophylaxis as resistant strains became prevalent. The development of sulfonamide-fast strains of the gonococcus has made sulfonamide therapy of gonorrhea practically worthless in the United States.

The following examples will suffice to point out that strains resistant to antibiotic substances can also be produced. At the beginning of an experiment 10 to 40 units of penicillin cured mice infected with the meningococcus, but after 25 passages through mice receiving subcurative doses, 1,400 units failed to cure. Dysentery bacilli which were inhibited in a medium containing 2 to 7 units of streptomycin per milliliter grew in the presence of 1,000 units per milliliter after 7 subcultures in media containing increasing amounts of streptomycin. Numerous strains of bacteria with marked resistance to other antibiotics have been reported.

Drug tolerance of bacteria is fairly specific. Development of resistance to a sulfonamide drug does not influence the sensitivity of an organism to penicillin. In certain instances resistance to one drug extends to other closely related compounds, but often the specificity of tolerance is such that where one sulfonamide will not inhibit the growth of a bacterium another one will. If strong drug-fastness is established in a strain, this property may be maintained for many generations, even when the organism is grown in the absence of the drug. There is also some evidence that drug-fast variants occur which have had no previous exposure to the drug in question. Resistant strains appear to arise as spontaneous mutations or as organisms with adaptive enzymes which can function in the presence of the drug.

## 18

### MICROBIAL ASSOCIATIONS— SYNERGISM AND ANTIBIOSIS

What knowledge we have of the behavior of bacteria has been gained almost entirely from pure culture studies. This means that the descendants of one bacterium have been studied and that, separated from all other organisms, they behave in a certain way. But rarely are bacteria found isolated from each other in nature. In soil and water a variety of species, saprophytes and autotrophs, aerobes and anaerobes, proteolytic and fermentative, pigmented and colorless vie with each other, with other microorganisms, and with the elements to survive and propagate their kind.

As for all organisms, life for the bacteria is a struggle for existence. In the struggle some bacteria, due to their particular requirements and metabolic activities, help each other; their association is one of symbiosis. More commonly bacteria of different inheritance get along amicably enough so that they can live together in the same community. On the other hand, the competition between two different species for food, space and the like may result in the more adaptable one over-growing the other, or in one inhibiting the growth of another even killing the second organism. Such an association is termed **bacterial antagonism** or **antibiosis**. For each kind of bacterium living in a mixed culture the situation resulting from their interrelations becomes that much more complicated. Not only among the free-living bacteria do these associations prevail but also the parasites have their advantages and disadvantages in trying to establish themselves in the body of a host. The successful members in each location, the ones which constitute the "normal flora" of that region, are those that can live in harmony with each other and with the host organism.

Although little attention has been paid to the interrelations of bacteria in mixed cultures, even beginning students wonder how obligate anaerobes flourish in shallow soil or around the teeth and gums. The obvious explanation here is that the aerobes are continuously using up the free oxygen and thus creating an environment compatible with anaerobiosis. Whether this is the whole story or not is beside the point. The lesson to learn is that when growing with others in nature, bacteria can do what they cannot do in pure cultures in the laboratory. A pure culture of *Nitrobacter* cannot produce nitrates from protein, but in the soil the project of protein decomposition and nitrate production is a community



prise in which the *Nitrobacter* participates. The proteolytic enzymes of heterotrophs begin the job and the autotrophic nitrifiers finish it. Decomposition is initiated by digestive enzymes secreted by the bacteria into their surroundings. These secreted enzymes are common property, and the substances digested are available to all bacteria in the neighborhood. It is not difficult to understand that the enzymes of one bacterium may only partially decompose a substance that the enzymes of another, although unable to attack the original sub-



Fig. 126. Satellite phenomenon in mixed cultures of bacteria on agar containing sulfonamide compounds. In each case a bacterium susceptible to the concentration of sulfonamide compound in the medium was inoculated over the entire plate, and a sulfonamide-resistant organism was inoculated at one or more spots on the surface. Growth of the sulfonamide-susceptible bacterium occurred only around colonies of resistant organisms which produced sulfonamide-inhibiting substances.

(Left) Satellite colonies of *Streptococcus pyogenes* around a large colony of *Staphylococcus aureus*.

(Right) Satellite colonies of *Staphylococcus aureus* around a large colony of *Salmonella typhimurium*.

(From Pike and Foster: *J. Bact.* 47:97, 1944.)

ence, may act on the decomposition products of the first. In mixed cultures the enzyme systems of one bacterium may supplement the enzyme systems of another.

There are a number of instances on record in which two or more species working together can do what neither one of these species can do alone. This type of bacterial association is termed **synergism**. One of the lactic acid bacteria and *Clostridium chauvei* growing together can ferment glucose to butyl alcohol which neither one can produce alone. Certain sugars are decomposed to alcohol and gas by a mixture of two bacterial species, a fermentative reaction not accomplished by either species in pure culture. Other reports cite examples of inhibition of the normal activity of a bacterium due to the metabolic products of a second organism in the same culture. The presence of certain pathogenic

bacteria may decidedly influence the disease-producing power of another. For example, mixed infections with the diphtheria bacillus and staphylococci or with the diphtheria bacillus and Friedländer's bacillus are extremely mild, whereas infections in which the diphtheria bacillus is accompanied by certain streptococci are more severe than an uncomplicated case of diphtheria. The synergistic action

of these and other bacteria growing together in the body is offered as an explanation of their altered virulence.

Microorganisms often produce substances which promote or inhibit the growth of their neighbors. A growth factor may be synthesized by one organism and not by another although it may be essential to both. The so-called "satellite phenomenon" is an illustration of the production by staphylococci of a growth-promoting substance, known as the V factor, needed by the influenza bacillus. If the entire surface of a plate containing medium complete in all respects except the V factor is inoculated with the influenza bacillus and then *Staphylococcus* is introduced onto one spot only, no growth of the influenza bacillus will be found except around the *Staphylococcus* colonies. Undoubtedly many species in mixed microbial populations are dependent on their associates for such growth-promoting material (Fig. 126).

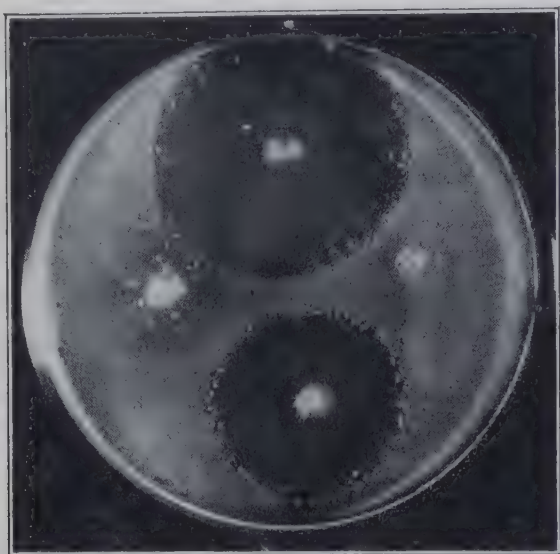


Fig. 127. Antibiotic activity among enteric bacteria. Plate was originally inoculated at four points with four different enteric organisms. When colonies developed the cells were killed by exposure to chloroform vapors. Then the entire plate was inoculated with *Escherichia coli*. The resulting plate shows that two organisms were markedly antibiotic, one was slightly and another not antibiotic against *Escherichia coli*. (From Fredericq, P., and Levine, M.: *J. Bact.* 54:785, 1947.)

In recent years an enormous amount of investigation has centered on the subject of microbic antagonism. It has long been known that water and soil microorganisms are natural foes of certain pathogenic bacteria. Studies have shown that while the typhoid bacillus survives in sterile water for from 15 to 25 days it lives only 1 to 4 days in raw river or canal water. That the unfavorable influence is due to living microorganisms was shown in reports stating that the same bacillus survived from 20 to 51 days in polluted water after it had been sterilized and died after 9 to 13 days in unsterilized surface water. The higher the degree of pollution of the water, *i.e.*, the greater the number and kinds of microorganisms present, the shorter is the survival time of the typhoid bacillus in the water. The same is true for its survival in soil, as well as for that of the bacilli of tuberculosis, Malta fever, and other pathogens in soil and water. Certain soils are more antagonistic than others depending on the types of saprophytic bacteria present and the type of bacterium subjected to



and purified before testing its usefulness in the treatment of disease. To be an effective therapeutic agent in the human or animal body an antibiotic must be nontoxic to the body, its action must not be hindered by tissue substances or enzymes, and it must inhibit the growth or cause the death of the infecting microorganism. Table S includes some proven and promising antibiotics of bacterial and mold origin.

## Antimicrobial Methods

### 19

## BACTERIOSTASIS AND DISINFECTION

Bacteria multiply only under circumstances making for a favorable environment. If one factor in the environment is sufficiently deranged, the harmony between the organism and its surroundings is thrown off balance, with the result that the organism either ceases to grow and reproduce or it dies. Man puts this fact to work in his fight to control microorganisms that cause disease, spoil his food and threaten his welfare in other ways. He deliberately exposes them to conditions which inhibit their growth or cause their death. The agents and methods employed are named according to the ends they accomplish.

**Sterilization** is a process that frees a substance or an article from all living organisms. There are no degrees of sterility. Only when every last organism, pathogenic and nonpathogenic, has been killed or removed has sterilization been accomplished.

An agent that kills microorganisms is known as a **germicide**, while the terms **bactericide**, **virucide** and **fungicide** are restricted to agents which destroy bacteria, viruses and fungi respectively. **Disinfection** is any process that kills disease-producing microorganisms in a relatively short time, and a **disinfectant** is an agent that is used to accomplish disinfection. Either a physical factor such as heat or a substance like lysol may be a disinfectant. Disinfection of a pure culture of *S. typhosa* also results in sterilization of the culture, but in the disinfection of feces from a typhoid patient the typhoid bacillus is destroyed and, although nonpathogens of the same resistance as *S. typhosa* may die as well, other organisms survive. Disinfection of materials harboring bacterial spores such as those of gas gangrene and the tetanus bacillus generally amounts to sterilization since spores are the most resistant forms of life.

**Bacteriostasis** is a state that prevents the multiplication of bacteria. Desiccation, cold and certain concentrations of dyes and other chemicals are **bacteriostatic** agents since they retard the life processes of the bacteria but do not kill them. Held thus in a state of suspended animation, bacteria may be preserved for considerable time though continuous inhibition will eventually end in death.

**Antisepsis** in its true sense means the same as bacteriostasis, and the adjective **antiseptic** is synonymous with bacteriostatic. However, both are popular expressions used to denote conditions or substances exerting either bacterio-



tic effect or disinfecting action. The terms antiseptics and antiseptic serve no purpose in modern scientific language.

Whether death or inhibition is the outcome of an antimicrobial method usually depends on the degree of injury to the organisms rather than the kind of injury. For instance, a certain temperature is optimum for the growth of each bacterium. A rise in temperature slows growth and a degree above the maximum temperature permits no growth although the organisms remain alive. With further increase a degree of heat is reached that will kill the bacteria in a short time. The same is true when a harmful chemical agent is introduced into the organism's environment. A certain concentration of the substance may kill the bacteria in a matter of minutes, whereas the same substance diluted will simply prevent growth and multiplication. Moreover, different bacteria (and other microorganisms) are not equally sensitive to the same treatment. A procedure that kills one organism may only inhibit another.

Bacteria must be exposed continuously to the bacteriostatic agent if growth is to be suppressed. As soon as the inhibitory agent is withdrawn (or the bacteria are removed from its influence) they will begin to grow and multiply. To determine whether an agent is lethal and not merely inhibitory the organisms are placed in contact with the agent and are then transferred to favorable conditions. In testing the action of heat, for example, recently inoculated cultures are exposed to high temperatures and after a suitable time they are incubated at their optimum temperature. In the case of a substance such as phenol the organisms are introduced into a solution of known concentration and samples are removed from time to time to be incubated in a nutrient medium. Growth or no growth in the cultures will indicate whether or not the organisms have been killed.

It is convenient to consider separately the physical and chemical agents of bacteriostasis and disinfection.

## PHYSICAL AGENTS

**Light.** Direct sunlight is a powerful bactericide and even diffuse daylight is inhibitory to the growth of most bacteria. Not all the sun's rays are bactericidal, but its lethal action is due almost entirely to the ultraviolet rays of the spectrum which have wave lengths ranging from about 2000 to 2800 Ångström units (Fig. 129). Of these the rays most active in killing bacteria and other microorganisms fall into a range from 2600 to 2700 Ångström units. Quartz mercury vapor lamps (not to be confused with ordinary "sun lamps") are an excellent source of such lethal radiations. Ultraviolet rays have little or no power of penetration; they cannot pass through ordinary window glass, fabrics, body surfaces, or dirt on floors and other surfaces. When sunlight is cut off by clouds or smoke its bactericidal property is greatly diminished. The most resistant vegetative bacteria, such as the tubercle bacillus, and even bacterial spores are killed by several hours of direct sunlight, whereas they may live for weeks or months in the dark. There seems to be little difference between the

resistance of the vegetative cells of bacteria and their spores when they are exposed to ultraviolet rays. The length of exposure necessary to kill bacteria depends on the intensity of the light and on the sensitivity of the organism. Clothing, mattresses, pillows and other articles that cannot be disinfected conveniently otherwise are exposed to outdoor sunlight for at least 6 to 8 hours. Care must be taken to turn these articles in order to subject all surfaces to the sun's rays.

Ultraviolet irradiation is an excellent way to disinfect the air. Modern hospitals use this method to reduce the chances of infection in operating rooms.

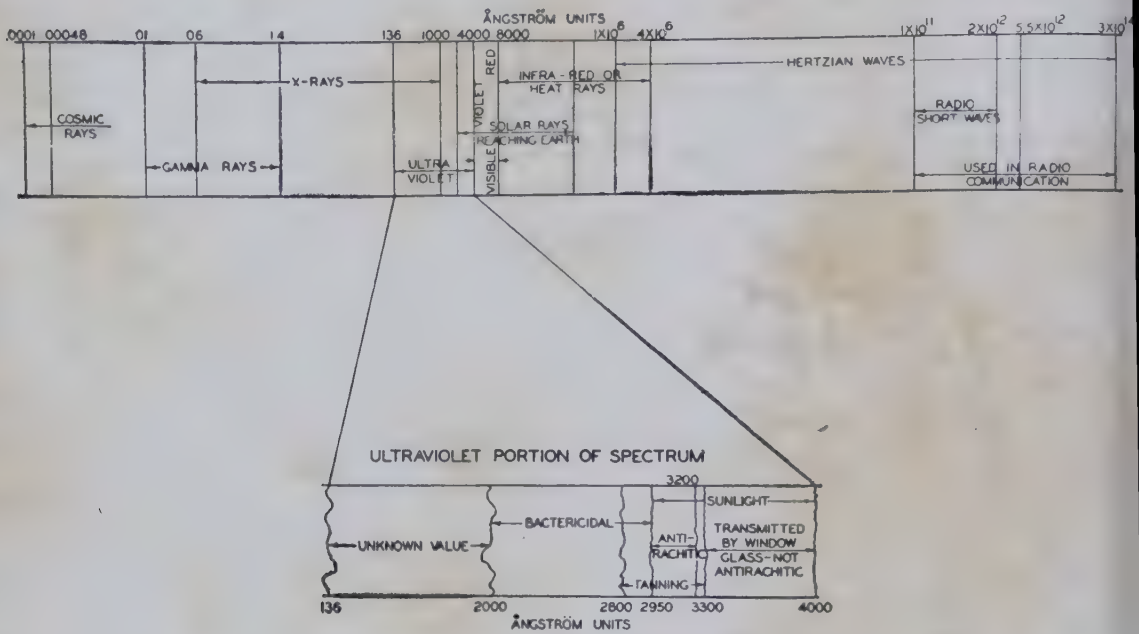


Fig. 129. The ether spectrum. (Courtesy of Westinghouse Electric Corp.)

nurseries and wards. In children's wards, where ultraviolet light is arranged to form a barrier or curtain of bactericidal rays around each bed or cubicle, cross-infections with the acute respiratory diseases have been greatly reduced. At present ultraviolet light has only limited application in protecting the public health. It is employed to prevent the spoilage of certain foods, particularly bakery goods and meats. Irradiation of milk to increase its vitamin D content should not be confused with disinfection. Establishments serving the public are apt to overrate the disinfecting power of ultraviolet lamps. Irradiation of drinking glasses and toilets, for example, does not guarantee their safety, since only the surfaces directly exposed are affected, often the time of exposure after contamination is too brief and not all the so-called ultraviolet light bulbs are efficient generators of the lethal rays.

**Cold.** Bacteria are very resistant to cold. The only effect of low temperatures on most bacteria is to slow down the chemical reactions in the cells and thus inhibit their growth. Existing at a low metabolic rate bacteria survive longer in the cold than at favorable growth temperatures. If quickly frozen and held at



very low temperatures, say around  $-70^{\circ}\text{C}$ , bacteria, rickettsiae and filtrable viruses may be preserved for many months.

The bacteriostatic action of cold is most widely and successfully applied in the prevention of food spoilage. Refrigeration delays decomposition as long as the microorganisms are kept from growing. A few saprophytic bacteria can grow over long periods at low temperatures and certain molds are quite adaptable to cold. However, neither cold nor freezing acts as a disinfectant. Foods contaminated with pathogenic bacteria are usually no safer after cold storage or storage in deep freeze lockers. Ice cream experimentally inoculated with *S. typhosa* has yielded live typhoid bacilli after storage at  $-4^{\circ}\text{C}$  for over two years. Intestinal organisms survive for months in ice made from contaminated water. Slow freezing has a different effect on bacteria than quick freezing. In the slow process large ice crystals are formed which mechanically disrupt and thus destroy a number of bacteria, but freezing never sterilizes water or foods, and pathogens as well as nonpathogens may survive. The presence of colloids, such as fats and proteins, greatly reduces the chance of destroying bacteria by ice crystals. In quick freezing the destructive ice crystals are not formed.

**Drying.** Enzymes cannot act without moisture, and, therefore, desiccation arrests microbic growth. In the dry state delicate bacteria may live not more than an hour, while bacterial spores may survive for years. Resistance of the bacteria depends on a number of factors, including the nature of the bacteria and how many of them are present, the rapidity of the drying process, temperature, concentration of oxygen in the environment and the kind of material in which the bacteria are suspended. A combination of light and drying is more effective in destroying bacteria than drying in the dark. Slow drying kills more bacteria than quick drying. The lower the temperature, the fewer organisms die when deprived of moisture. If an aqueous suspension of bacteria is frozen rapidly and the frozen mass is dried by high vacuum distillation, the dried bacteria held in vacuum will remain viable for years. This method, known as the lyophil process or lyophilization, is often used in preserving stock cultures, bacterial products, such as enzymes and toxins, and filtrable viruses. Pathogenic bacteria dried in feces, sputum, blood, pus and other exudates are protected from complete desiccation by a film of organic material, and they remain alive longer than if dried when suspended in urine, water or saline. Organic matter coats the bacteria and droplets of respiratory secretion expelled into the air by coughing and sneezing and thereby prolongs the danger from these dried secretions. The more concentrated the suspension of bacteria, the longer it will take for drying to kill them.

Because of the great influence exerted by factors such as these, it is impossible to state the exact length of time a particular bacterium can survive drying. Nevertheless, bacteria can be arranged in a series according to their survival time when allowed to dry in air, soil, dust, and on floors and objects. Bacterial spores are the most resistant. There are reports of anthrax spores withstanding up to 40 years of drying. The tubercle bacillus, probably owing to its high lipid content, survives drying longer than other nonsporulating bacteria. It has been

recovered alive in dried sputum after six to eight months. Staphylococci can be cultivated from pus which has been dried for months. Hemolytic streptococci have been found to be virulent after two weeks in dust. More fragile organisms such as the whooping cough bacillus may survive in dried respiratory exudate no longer than a day or two, while the gonococcus and meningococcus die in a matter of an hour or two and the spirochete of syphilis is even more quickly killed by drying.

**Unfavorable Osmotic Pressure.** Bacteria are much more resistant to unfavorable osmotic pressures than most other cells. Nevertheless, high concentrations of nontoxic salts and sugar exert a strong bacteriostatic effect either by causing water to leave the cells (plasmolysis) or perhaps by dehydrating the proteins in the medium. Many foods are preserved in salt brines, and salting is often combined with other inhibitory agents as in dried salt fish and smoke meats. The keeping quality of jellies and preserves depends upon the inhibitory action of their high sugar content.

**Dry Heat.** Although excellent as a sterilizing agent, dry heat is rarely used to disinfect infectious materials. It is impractical except possibly in emergencies when other methods are not available. Hot air cabinets are satisfactory for killing pathogens which may contaminate dairy equipment and food utensils as well as in reducing the number of saprophytic microorganisms on their surfaces. Oven baking of foods lessens the chances of food-borne infection enormously, but there is no guarantee that a contaminated food is thus rendered harmless. In experiments to prove this point, the nonsporulating colon bacillus has been recovered from the center of baked dishes which a cook would pronounce "done." The explanation offered for survival of bacteria at oven temperatures of  $325^{\circ}$  to  $400^{\circ}$  F is that the temperature of moist foods does not rise above the boiling point, and that, since semisolid and solid foods are poor conductors of heat, the center of a dish is not heated to the thermal death point of the contaminating bacteria.

Incineration is the best way to kill pathogens in refuse and, at the same time, to eradicate objectionable materials such as soiled dressings, used paper sputum cups and paper tissues contaminated with upper respiratory discharges.

**Moist Heat.** Cellular proteins are coagulated by moist heat and once this has occurred the cells are dead. The excellence of moist heat as a bactericide is verified by the fact that it is the most commonly used agent of sterilization. It is also widely employed as a disinfectant and sometimes is the only satisfactory one. The thermal death point of most nonsporulating bacteria is  $55^{\circ}$  to  $65^{\circ}$  C for 10 minutes, *i.e.*, a 24-hour pure culture of the organisms growing in a nutrient broth of pH 7 is sterilized at this time and temperature. If the time is shortened a higher temperature will be necessary, while a lower temperature may accomplish the same thing if the time is prolonged. Several other conditions influence the action of moist heat and an important one is the presence of organic matter that protects the bacteria. Actually the pathogens to be killed are generally in organic matter, in the discharges and excreta of the patient, in the soil of used



es, silverware, towels, bed linen and other articles contaminated by the patient. Therefore in practice the temperature is raised above that of the thermal death point to insure disinfection. Boiling or treatment with live steam for ten minutes is used to kill nonsporulating bacteria on contaminated articles, but autoclaving is recommended for infective discharges and it is the only sure way to destroy the spores of the gas gangrene, tetanus and anthrax organisms. Moist heat is more reliable than chemical disinfectants in killing acid-fast bacilli such as *Mycobacterium tuberculosis*.

Cooking, especially boiling, kills many microorganisms in foods and is probably the most effective single measure in preventing food-borne disease. In preserving foods by canning the aim is sterilization, but what is usually achieved is a sort of super-disinfection. Almost all the organisms are killed by the canning process and the few that remain alive are generally aerobes that cannot multiply in hermetically sealed containers. Processing in efficient pressure cookers (in which steam is compressed just as it is in the autoclave to raise the temperature above the boiling point) is the only reliable way of destroying bacterial spores. The spores of *Clostridium botulinum*, the anaerobe that causes a highly fatal food poisoning known as botulism, may survive hours of ordinary boiling.

The temperature of water used in washing dishes by hand is not hot enough for disinfection. Rinsing with boiling water is a valuable practice in reducing the numbers of bacteria, but the time element here is not long enough to insure disinfection. Dishes, silverware and glasses known to be contaminated should be boiled or exposed to live steam for 10 minutes. In public dining places all eating utensils should be disinfected, preferably by scalding water in properly operated mechanical dishwashers.

Pasteurization of milk and milk products is disinfection by moist heat with the temperature and time factors set to kill the most resistant disease-producing microorganism that may be present. Pasteurization destroys the tubercle bacillus along with other less hardy pathogens including the typhoid bacillus, the *Brucella* of undulant fever and hemolytic streptococci. The United States Public Health Service recommends heating raw milk to at least 143° F (61.7° C) for 30 minutes or to at least 160° F (71.1° C) for 15 seconds.

## CHEMICAL AGENTS

Chemical bactericides are poor agents of sterilization compared with steam under pressure or the high temperatures of hot air ovens. Although in many hospitals sharp instruments intended for minor surgery are sterilized by soaking in solutions of Amphyl, Zephiran chloride, formaldehyde-alcohol or some other substance, the method is not recommended. "Cold sterilization" is quite satisfactory for killing nonsporulating bacteria, but it is not effective in destroying bacterial spores. The great usefulness of the chemical agents lies in their disinfecting action. They are especially effective and convenient in killing pathogenic microorganisms in exudates, excreta and other wastes from infected patients. Only

a small number of antibacterial substances can be used therapeutically in body and their curative effect generally lies in their ability to inhibit micro-growth rather than in their disinfecting action. Powerful cheap chemical disinfectants are utilized to destroy pathogens which have spread into the environment.

**Acids.** Strong mineral acids such as hydrochloric, sulfuric and nitric acids are highly bactericidal, but their use as disinfectants is impractical because they are dangerous to man and animals as well as destructive to fabrics, metals and other materials. Their bactericidal action is proportional to the degree of dissociation, *i.e.*, the higher the concentration of free hydrogen ions in solution, the greater its lethal action. Weak organic acids have little or no disinfecting power, but are bacteriostatic since a hydrogen ion concentration greater than that of  $pH\ 7$  is inhibitory to most bacteria. The United States Food and Drug Administration allows some mild acids and their salts, for example benzoic acid and sodium benzoate, to be added to certain foods as preservatives. The keeping quality of food pickled in vinegar depends on its acetic acid content. Undiluted vinegar which usually contains 5 per cent or more acetic acid is lethal to vegetative bacteria in a relatively short time, but the concentrations used in foods are only bacteriostatic. Boric acid as used in the irrigation of mucous membranes has little if any direct effect on bacteria; even a saturated aqueous solution will not destroy the staphylococci.

**Alkalis.** The bactericidal action of the hydroxides depends chiefly on the concentration of free hydroxyl ( $OH^-$ ) ions released when they are in solution. Gram-negative, nonsporulating bacilli are highly susceptible to alkalis, while gram-positive bacteria are slightly more resistant. Bacterial spores are not readily killed by any chemical agents, but the alkalis are more efficient than most. The weak substances are not satisfactory disinfectants against the tubercle bacillus. Otherwise strong alkalis are excellent bactericides, but because they are also strong poisons their usefulness is restricted to the disinfection of excreta and of outdoor buildings such as barns and chicken houses. Household ammonia, borax and sodium carbonate have no practical value as disinfectants.

**Sodium Hydroxide.** Household lye which is about 98 per cent sodium hydroxide is at least ten times as efficient as phenol in killing the typhoid bacillus. A 5 per cent solution of lye is recommended to destroy anthrax spores where heat cannot be used and weaker solutions are lethal to nonsporulating bacteria. Beerage and milk bottles are often disinfected by hot caustic soda ( $NaOH$ ) solutions.

**Calcium Hydroxide.** When quicklime (calcium oxide,  $CaO$ ) is dissolved in water, calcium hydroxide ( $Ca(OH)_2$ ) or slaked lime is formed. Freshly prepared slaked lime, either in the form of a paste known as milk of lime or the more dilute whitewash, is a vigorous bactericide. It is important that the quicklime has not been exposed to the air, for when this happens moisture and carbon dioxide are gradually absorbed and calcium carbonate ( $CaCO_3$ ), a substance of no bactericidal value, is formed. The calcium hydroxide of slaked lime left in contact with air for any time will also be converted into the inert calcium carbonate.



bonate. Dusts containing quicklime are bactericidal only if they fall where there is moisture enough to dissolve them. Whitewash made from substances other than quicklime have little or no disinfecting power.

**Phenol, Cresol and Related Compounds.** Crude carbolic acid and natural cresol are impure products formed by the distillation of coal tar. Both they and the purified substances extracted from them, phenol (pure carbolic acid) and the three forms of cresol (ortho-, meta- and para-cresol) are excellent bactericides.

**Phenol.** Lister first demonstrated the capable performance of phenol in anti-septic surgery and it has since become a standard by which other disinfectants are compared. Most vegetative bacteria are killed by from 1:80 to 1:110 dilutions in 5 to 10 minutes, but spores are much more resistant. Anthrax spores, for example, are not destroyed after 24 hours in 5 per cent phenol. The poor solubility of phenol prohibits solutions of much higher concentrations. One great advantage of phenol is that organic matter has little influence on its bactericidal action, and therefore it is a good disinfectant for feces and exudates. A 5 per cent solution of either crude carbolic acid or phenol is commonly used to disinfect excreta, sputum, instruments, utensils, linens and the like. Owing to its toxicity for living tissues, even dilute solutions of phenol are rarely used on the body.

**Resorcinol.** Certain derivatives of resorcinol (hydroxy phenol) are actively bactericidal. One of these, **hexylresorcinol** which is sold in a solution of glycerin and water labelled S.T.37, is remarkable for low tissue toxicity. In laboratory tests it is over a hundred times more potent as a disinfectant than phenol, but in actual use the presence of organic debris easily protects bacteria from its action.

**Halogen Derivatives of Phenol.** If chlorine or bromine is introduced into phenolic compounds the bactericidal power of the resulting substance is greater than that of phenol. **Amphyl** is a mixture of phenol derivatives, one of which is a chlorine compound. Employed in 0.5 to 2 per cent solutions it is a valuable disinfectant.

**Cresol.** The cresols are more potent bactericides and are less irritating to tissues than phenol. Their greater insolubility is a disadvantage, but mixed with liquid soaps and alkalis they form emulsions which are good cleansing agents as well as general disinfectants. Many cresol compounds are available under various trade names. *Liquor Cresolis Saponatus*, an emulsion of cresol in potash-vegetable oil soap, is official in the United States Pharmacopoeia. *Lysol* is a mixture of cresol and potash soap with alcohol. Most pathogens are killed by cresol compounds diluted from 1:50 to 1:500 at the end of 10 or 15 minutes, but they cannot be relied upon to eliminate the spores of the gas gangrene, tetanus and anthrax bacilli. The saponified cresol compounds used in 2 to 5 per cent solutions are excellent bactericides for excreta, utensils, instruments and other articles, and combined with plenty of elbow grease they cleanse and disinfect floors, toilets, sinks and other contaminated surfaces.

**Alcohol.** Absolute (100 per cent) ethyl alcohol has little or no bactericidal power. Maximum lethal effect is obtained with 50 to 70 per cent aqueous solutions, and as the concentration of alcohol is decreased or increased beyond this range its bactericidal efficiency rapidly diminishes. Alcohol kills bacteria by coagulating their cellular proteins, and one reason why it is not more useful as a disinfectant is that it also coagulates body proteins, often leaving unharmed the bacteria embedded in exudates and other organic matter. Since alcohol evaporates quickly it has little time to act when wiped over the surface of skin or instruments. Superficial dabbing of the skin with alcohol preliminary to hypodermic injection probably accomplishes very little in the way of disinfection. Most likely infection fails to follow because of the absence of true pathogens on the skin and the natural resistance of the tissues to bacteria which may enter with the puncture. If it is to do any good the alcohol swab should be applied with sufficient vigor and long enough to **cleanse** the area. Soap and water scrub is just as effective and acetone is superior as a cleansing agent. Where longer exposure is allowed more satisfactory results are obtained. Soaking in 70 per cent alcohol reduces the number of bacteria on hands and arms after presurgical scrub. Oral thermometers are disinfected if kept in alcohol which has not deteriorated to less than bactericidal strength.

Alcohol is at best a mild disinfectant that can be relied upon to kill only the more sensitive organisms such as those of the respiratory tract. Under no conditions should it be used to disinfect contaminated instruments. In the preservation of biological specimens alcohol serves as a bacteriostatic agent by checking the growth of resistant bacteria. Ninety-eight per cent isopropyl alcohol is reported as having twice the bactericidal strength of 70 per cent ethyl alcohol and being less corrosive.

**Formaldehyde.** Formalin (**Liquor Formaldehydi**) is a solution of not less than 37 per cent formaldehyde gas. It surpasses phenol as a bactericide, but has certain disadvantages. Dilutions of formalin are unstable since the active ingredient, formaldehyde, gradually volatilizes. Formaldehyde fumes are highly irritating to the mucous membranes of eyes and respiratory tract and formaldehyde solutions harden tissues. On the other hand, organic matter interferes only slightly with its action, and a 10 per cent solution of formalin is a satisfactory disinfectant for sputum, feces, urine, clothing and many other materials. Formalin is more effective in killing spores than phenol, but it is not to be trusted as a sterilizing agent for surgical instruments and gloves. Formaldehyde fumes are sometimes used as an adjunct to autoclaving in the sterilization of dressings. Gaseous disinfection with formaldehyde is discussed under the subject of fumigation (page 226).

**Halogens. Chlorine.** The bactericidal power of free chlorine against most bacteria is around one to two hundred times as great as that of phenol, and due to its action as an oxidizing agent together with the toxicity of chlorine ions for protoplasm. In its various forms chlorine is the most widely used chemical



of disinfection. Chlorine gas and liquid chlorine are employed in the disinfection of metropolitan water supplies and sewage. Elsewhere chlorine compounds, namely the **hypochlorites** and **chloramines**, are more convenient and safer to handle.

**Calcium hypochlorite** ( $\text{CaOCl}_2$ ), commonly known as chlorinated lime, bleaching powder and chloride of lime, is made by treating lime ( $\text{CaO}$ ) with chlorine gas. The resulting powder usually contains from 25 to 30 per cent chlorine. Solutions of hypochlorites deteriorate rapidly and the germicidal power of hypochlorite powders declines as they age. However, this very property of instability endows these substances with bactericidal power, for the disinfecting strength of the compound depends on the amount of chlorine liberated as it decomposes. Hypochlorites combine readily with organic matter and an excess amount of chlorinated lime must be used to disinfect materials like feces and urine. Whereas a million gallons of clear water may be disinfected by eight pounds of chlorinated lime, a final concentration of 1:10 is recommended for the disinfection of feces. The action of hypochlorites is practically instantaneous and terminates immediately, thus making these solutions suitable for rinses to disinfect the **clean** surfaces of glasses, dishes and other utensils. Disadvantages of chlorinated lime include its corrosive action on tissues, metals and other materials, and its ineffectiveness in killing acid-fast bacteria such as the tubercle bacillus. Nevertheless, the low cost of chlorinated lime and its strong germicidal action for all vegetative bacteria except acid-fast bacilli make it a good disinfectant for general use.

**Sodium hypochlorite** ( $\text{NaOCl}$ ) is a potent bactericide formed by the action of chlorine on caustic soda ( $\text{NaOH}$ ). In solution it is used for the disinfection of restaurant and dairy equipment and is sold as laundry bleaches such as Clorox, B-K, HTH-15 and Diversol are hypochlorite products. Manufacturers of these and other proprietary chlorine compounds provide information on the amount of available chlorine in the products and the concentrations which are effective for various purposes. In World War I **Dakin's solution**, a preparation containing sodium carbonate, chlorinated lime and boric acid, was found to be effective in controlling wound infections without undue tissue irritation. It did, however, retard healing and with the advent of the sulfonamide drugs its use was largely discontinued.

A number of chlorine-containing, complex organic compounds known as **chloramines** offer several advantages over the hypochlorites, namely, they are less irritating to tissues and less corrosive in general, are more stable which allows for prolonged bactericidal action, and have slight affinity for organic matter. **Chloramine-T** or **chlorazene**, **Dichloramine-T** and **Azochloramid** have been employed in the treatment of infected wounds and mucous membranes. Combined with the sulfonamides, Azochloramid is said to fortify and prolong their bacteriostatic effect in the wound. Utensils and equipment, particularly in food and milk plants, can be disinfected by soaking in a chloramine solution, but, owing to their slow rate of liberating chlorine, they are unsatisfactory as rinses.

Water containing a high percentage of suspended matter is sometimes chlorinated by means of a chloramine.

**Iodine.** When applied to the skin iodine kills most of the bacteria it reaches in a matter of seconds, but like other disinfectants it cannot sterilize the skin. **Tincture of iodine** (an alcoholic solution of metallic iodine and potassium iodide) is more effective as a skin disinfectant than an aqueous preparation such as **Lugol's solution**. A serious objection to iodine is its danger as a tissue irritant; prolonged, and in sensitive persons even a brief, contact with the skin results in blisters or "iodine burn." Like chlorine, iodine exerts its bactericidal effect by acting as a protoplasmic poison as well as an oxidizing agent. A mixture of tincture of iodine and glycerin is sometimes applied to infected oral mucous membranes, but its action is confined to exposed surfaces since there is little or no penetration to embedded organisms.

**Salts of Heavy Metals.** Salts of mercury and silver have long been known as powerful antibacterial agents, but it is now recognized that their action is more bacteriostatic than bactericidal. Their effectiveness is due to the toxicity of the metal ion or of the whole molecule and does not depend on electrolytic behavior.

**Mercury.** **Bichloride of mercury** ( $\text{HgCl}_2$ ; corrosive sublimate), commonly employed as a disinfectant in dilutions of 1:1000 to 1:10,000, is bacteriostatic in even higher dilutions. Its usefulness is limited, however, because it is poisonous to man and animals, corrosive to metals and a strong coagulant of proteins. Bichloride of mercury should not be used as a disinfectant of organic matter, for the mercury ions combine with and precipitate the proteins in sputum, feces and other wastes, and are therefore prevented from contact with the bacteria embedded in these materials. To diminish the toxic, corrosive and protein-precipitating properties of the inorganic salts, complex organic mercury compounds have been developed, including **merthiolate**, **metaphen**, **mercurchrome** and **phenyl mercuric nitrate**. These compounds are used primarily as disinfectants of the skin and superficial wounds.

**Silver.** As in the case of the mercury compounds, silver salts are potent antibacterial substances owing to the toxicity of the metal ions and also to the **oligodynamic\*** action of the metallic silver which results from the reduction of silver ions. Not only is bacterial protoplasm poisoned by silver ions, but the protoplasm of higher organisms is also injured by them. The free silver ions of dissociated inorganic salts combine with and precipitate chlorides and proteins in tissues and exudates, although the silver-proteins thus formed are mildly bactericidal. Of the simple salts, **silver nitrate** ( $\text{AgNO}_3$ ) is the most widely used, the effective concentrations being in the range of 1:10,000 for bacteriostatic and 1:1000 for bactericidal action.

Credé introduced the practice of instilling 1 or 2 drops of 2 per cent silver

\* Oligodynamic (*oligo*, small; *dynamic*, powerful) refers to the lethal effect of extremely small amounts of certain metals on bacteria in water. Silver has the most powerful oligodynamic activity of the metals.



ate solution into the conjunctival sac of newborn babies in order to prevent ophthalmic infection of the eyes and subsequent blindness. As practiced today so-called Credé treatment (actually prophylaxis against gonorrheal ophthalmia neonatorum) employs a more dilute (1 per cent) solution of silver nitrate which is less irritating to the conjunctivae. Dentists apply ammoniacal silver nitrate  $[\text{Ag}(\text{NH}_3)_2\text{NO}_3]$  to cavities to prevent the growth of bacteria. Colloidal silver preparations, made by mixing finely divided metallic silver with silver-protein compounds, lack the corrosive and irritating properties of simple silver salts and yet retain a considerable capacity as bactericides. Furthermore, since there is only slight dissociation of colloidal silver compounds there are few silver ions liberated to combine with tissue chlorides and proteins. **Protargol**, **argyrol**, **argyn**, **silvol** and **neosilvol** are colloidal silver compounds of high bacteriostatic and some degree of bactericidal power.

**Oxidizing Agents.** Substances which liberate nascent oxygen are antibacterial owing to their activity as oxidizing agents. **Hydrogen peroxide** is such a substance. It rapidly deteriorates in the presence of certain inorganic catalysts and the enzyme catalase forming nascent oxygen and water. Nascent oxygen combines readily with organic matter including bacterial cell substance. The frothing observed with such satisfaction when commercial (3 per cent) hydrogen peroxide solution is applied to a cut is really the escape of oxygen and loss of the disintegrating element. Solutions of hydrogen peroxide decompose rapidly and, therefore, are unreliable for general use. One would expect anaerobic bacteria to be particularly susceptible to hydrogen peroxide, but its action is too mild and inefficient to destroy the spores of clostridia. It is said to be a potent disinfectant against certain nonsporulating anaerobes such as the oral spirochetes. It may have real value in wounds as a cleansing agent since the gassing loosens and helps remove dried blood and other tissue debris.

**Sodium perborate**, **zinc peroxide** and **benzoyl peroxide** are considered to be bacteriostatic rather than lethal for bacteria. These oxidizing agents have been used alone or with the sulfonamides in dressings of wounds and burns. Claims have been made for the efficiency of zinc peroxide and benzoyl peroxide in controlling the growth of anaerobic sporulating bacilli of gas gangrene. **Potassium permanganate** is a good bactericide, but there are so many objections to it including its deep purple stain that it is rarely used. **Potassium and sodium dichromate** combined with sulfuric acid in laboratory cleaning solution disinfect as they cleanse contaminated glassware. **Sodium azide** has a selective bacteriostatic action on gram-negative organisms. As previously mentioned, the effectiveness of **chlorine** depends in part on its action as an oxidizing agent.

**Soaps and Synthetic Detergents.** Ordinary handwashing regardless of the kind of soap used has little or no disinfecting value. Yet it is most important in controlling the spread of pathogenic microorganisms because the lather and scrubbing mechanically remove many bacteria as well as dirt. The thicker the lather and the longer the washing time the more bacteria are removed, and, incidentally, the greater the chance that less resistant organisms such as pneumococci and

streptococci may be killed in the process. There is no experimental proof that soaps advertised as "germicidal" or "antiseptic" have any more claim to the terms than ordinary soaps. Solutions of soaps will kill certain gram-positive bacteria if they are directly exposed for a matter of minutes, but bacteria in the pores of the skin may not be reached and the usual handwashing is terminated in less than one minute. The aim of surgical scrub is the removal of as many organisms as possible from the skin. This procedure followed by soaking in a disinfecting solution brings about maximum reduction of bacteria, but not sterilization of the skin.

Washing dishes by hand in warm soapy water is no guarantee that pathogenic organisms, if present, are killed or removed. Disinfection of dishes in public eating and drinking establishments must be accomplished by heat of a degree not tolerated by human hands or by chemical disinfection such as chlorination. The surfaces of dishes, glasses and silverware must be clean before chemical disinfection is effective.

In recent years industrial research has produced wetting and cleansing agents (detergents) which are in many ways superior to soaps. Certain of these **synthetic detergents** have proved to be bactericidal in high dilutions, thus offering promise as combination cleansing and disinfecting substances. The removal of grease and dirt from a surface involves primarily the wetting of the surface with a substance which will allow the complete displacement of the grease and dirt. Good **wetting agents** are substances which reduce surface tension; they are liquids which spread rapidly over a surface and remain in a stable layer once they are spread. Alcohol has a lower surface tension than water and is, therefore, a better wetting agent. In tincture of iodine and other tinctures it is the alcohol that increases their penetrating power over that of aqueous solutions. Soap lowers the surface tension of water and soapy water penetrates to surfaces not reached by water alone. The newer synthetic detergents, popularly known as "soapless soaps," reduce surface tension more efficiently than soap and also have the advantage of being stable in alkaline and acid solutions as well as not forming insoluble precipitates when used in hard water. The synthetic detergents, like soaps and other surface-tension reducing substances, fall into two classes: the **anionic detergents** which ionize with the active (hydrophobic) group as the electro-negative ion, and the **cationic detergents** which act by virtue of their positively charged ion.

Zephiran chloride (trade name for the quaternary ammonium compound alkyl dimethyl benzyl ammonium chloride) was the first of the cationic detergents to be recognized as a germicide. Reports of its bactericidal power state that a 1:20,000 dilution kills *Staphylococcus aureus* and *Salmonella typhosa* in 10 minutes and *Streptococcus hemolyticus* is destroyed in the same time by a 1:40,000 dilution, indicating that Zephiran chloride has about 200 times the disinfecting strength of phenol. Many other cationic detergents (chiefly quaternary ammonium salts such as Retarder LA, Emulsol 660B and Damol) have since been found to be bactericidal. Bacterial metabolism is inhibited by all cationic



detergents tested at a concentration of 1:3000 and several are active at 1:30,000. The anionic detergents do not possess the bacteriostatic or the disinfecting efficiency of the cationic compounds. Furthermore, while the cationic detergents are equally effective against gram-positive and gram-negative bacteria, anionic detergents such as Drene (triethanol amine lauryl sulfate) are selectively inhibitory and bactericidal for gram-positive bacteria only. Bactericidal concentrations of most synthetic detergents have little or no tissue toxicity. They are not greatly influenced by the presence of organic matter, but are inactivated by soaps and phospholipids.

TABLE 9. INHIBITING CONCENTRATIONS OF THREE TRIPHENYL METHANE DYES IN PEPTONE PHOSPHATE BROTH \*

	BRILLIANT GREEN	CRYSTAL VIOLET †	FUCHSIN (ACID)
GRAM-POSITIVE:			
<i>B. subtilis</i>	1:15,000,000	1:4,000,000	1:500,000
<i>S. ph. aureus</i>	1:4,000,000	1:1,000,000	1:300,000
GRAM-NEGATIVE:			
<i>S. paradysenteriae</i>	1:500,000	1:400,000	1:100,000
<i>S. typhosa</i>	1:510,000	1:85,000	1:12,000
<i>E. ch. coli</i>	1:550,000	1:100,000	1:12,000

\* Data from Kligler, I. J., A study of the antiseptic properties of certain organic compounds. *Exp. Med.*, 1918, 27:463.

† Crystal violet is one constituent of methyl violet which is, in turn, the most important component of gentian violet.

**Dyes.** The common substance in the modern synthetic dyes is aniline. By treating aniline in various ways the chemists have created thousands of pigments which form stable combinations with the proteins of silk, wool and other textiles. Many of these aniline dyes have selective affinities for the protoplasmic constituents of tissues, bacteria and other microorganisms. This has led to the development of differential staining (for example, Gram's method) and media such as Endo's basic fuchsin sulfite agar, eosin-methylene blue agar, crystal violet agar and brilliant green bile broth) for the selective cultivation of bacteria. Gram-positive bacteria are, in general, more sensitive to dyes, especially the basic dyes, than the gram-negative forms, and the most commonly used selective media contain a concentration of dye which inhibits the former but allows the latter to grow. Antibacterial action is associated with the chemical structure of the dye compound, *viz.*, the triphenyl methane dyes (including brilliant green, crystal violet and basic fuchsin) are highly bacteriostatic, and some of the acridine dyes (for example, acriflavine, acridine trypan blue and flavicide) are selective, whereas the azo dyes (such as Bismarck brown, chrysoidin, trypan blue and Congo red) have relatively slight inhibitory effect. Table 9 illustrates the powerful bacteriostatic action of three common triphenyl methane dyes on representative gram-positive and gram-negative bacteria. In greater concentrations such dyes are bactericidal for vegetative cells. Bacterial spores are not subject to their action; for instance, the spores of *B. subtilis* and *B. anthracis*

survive exposure to a saturated aqueous solution of gentian violet for hours at  $37^{\circ}$  C.

Certain dyes, such as gentian violet, proflavine and brilliant green, have been used on wounds and burns to prevent or control infections. Before introduction of sulfonamide drugs and antibiotics, infections of the genitourinary, upper respiratory and intestinal tracts were sometimes treated with methylene blue, trypanflavine or another of the less toxic dyes. Gentian violet is widely used as an antihelminthic against pinworms and other intestinal worms. Acriflavine is directed chiefly against certain pathogenic protozoa, the trypanosomes. There is recent evidence that certain dyes have antirickettsial activity. Methylene blue and toluidine blue have been shown to protect animals experimentally infected with the rickettsia of tsutsugamushi disease (scrub typhus).

**Fumigation.** The fumes of sulfur dioxide, chlorine and formaldehyde were once believed to be effective disinfectants of air and surfaces contaminated by patients with infectious diseases. Of necessity fumigation was performed as a terminal measure after the occupant of the sickroom had been removed, and incidentally, after the patient had usually passed through the most infectious stage of his illness. Sulfur dioxide and chlorine are not in themselves bactericidal but must combine with water to form sulfurous acid and hypochlorous acid respectively. Rarely is there enough moisture in the atmosphere to form effective concentrations of these acids, and the probability of air leaks minimizes the chance of maintaining these gases at bactericidal strength.

Moreover, if proper disinfection has been practiced during the patient's illness and if, on termination of the case, the sickroom is thoroughly cleansed, sunned and aired, fumigation is unnecessary. Today fumigation is directed against insects and vermin which harbor pathogenic bacteria, rickettsiae and viruses rather than against the microorganisms themselves. Hydrocyanic acid gas (HCN), a powerful insecticide as well as a deadly poison for man and animals, is used to destroy rats on board quarantined ships, eliminate vermin from warehouses and exterminate bedbugs and cockroaches from tenement buildings. Fumigants to control insects in the enclosed spaces of rooms, airplanes, etc., include sulfur fumes, pyrethrum sprays and DDT aerosol bombs. Modern disinfection utilizes ultraviolet irradiation, and the use of germicidal aerosol mists and vapors is in the experimental stage.

**Aerosol Mists and Vapors.** Lister first used a germicidal mist to control air-borne infection when he sprayed carbolic acid into the air of his operating room in an attempt to prevent infection of surgical wounds. However, the high toxicity of phenol makes it unsuitable as a sterilizing agent of air breathed by man and animals. In 1928 it was reported that a fine spray of sodium hypochlorite solution was nonirritating in a concentration lethal to colon bacilli suspended in air. Ten years later studies revealed that, if dispersed in air as very small droplets, certain germicidal agents, such as hexylresorcinol, were thousands of times more active in killing bacteria in air than the same agent in solution. The action of these germicidal aerosols was enhanced when they were



in propylene glycol or certain other glycols. Further investigation showed that propylene glycol alone was an effective germicidal aerosol, that glycol mists could be replaced by glycol vapors, and that triethylene glycol was superior to propylene glycol as a lethal agent against air-borne pathogens.

The glycol mists and vapors possess many properties of an ideal air disinfectant; they are powerfully germicidal, nonirritating, low in toxicity, odorless, tasteless and cheap. Air experimentally loaded with respiratory organisms, such as *beta* hemolytic streptococci, is sterilized within ten minutes by a concentration of one gram of triethylene glycol in 100 million ml. of air. Mice survive injections with pneumococci which have been suspended in propylene glycol-treated air, whereas control mice die after receiving pneumococci which have been similarly exposed to untreated air. In other tests propylene and triethylene glycol vapors protected mice placed in a test chamber which was sprayed with a suspension of influenza virus, but those exposed to the virus in the air of the control chamber succumbed to the infection. Glycol vapors are also effective in reducing experimental air-borne infections with certain other viruses.

Where triethylene glycol vapor has been put to a practical test, as in army barracks and isolation wards, it has effectively reduced the chance of air-borne respiratory infections. Whether a glycol vapor will be satisfactory for general disinfection and how it compares with ultraviolet irradiation as a practical means of air disinfection remains to be seen. The germicidal action of the glycol vapors is greatly influenced by the temperature and the amount of moisture in the air. Another influential factor is the amount of particulate matter in the air, and these suppression measures must be combined with the use of glycol vapor for effective control of air-borne pathogens. The cumulative effect of prolonged exposure to air containing glycol vapors on man is not known. However, rats and monkeys living continuously in the presence of germicidal concentrations of propylene or triethylene glycol mist show no ill effects.

## FACTORS INFLUENCING THE ACTION OF A DISINFECTANT

Not all the bacteria in a pure culture are killed simultaneously by a disinfectant, but a certain proportion of cells die in each unit of time. If 90 per cent are destroyed in the first minute of exposure to the bactericidal agent, then 10 per cent of the survivors will die in the second minute and so on until all are killed or, theoretically, less than one bacterium remains alive. The greater the number of bacteria to be killed the longer the time needed to complete the disinfecting process. The concentration of the disinfectant influences the speed of the bactericidal action, but it does not follow that if one doubles the concentration disinfection will be complete in half the time. In general, the greater the concentration of a bactericidal substance, the degree of heat or the intensity of lethal rays, the less time required for disinfection. However, there is a limit at which increasing concentrations of a bactericidal substance do not result in accelerated disinfection, and may even be less efficient than more dilute

solutions. For example, 70 per cent ethyl alcohol is bactericidal whereas absolute ethyl alcohol is not.

Obviously, the **time of exposure** to the disinfectant is an important factor. The longer the bacteria are in contact with the bactericidal agent the more will be killed, and a low concentration acting over a long period of time may be as effective as a higher concentration of the same agent during a short exposure. With few exceptions the disinfecting rate of chemical agents is accelerated by increase in **temperature**. For instance, in one experiment a 1:75 dilution of phenol sterilized a culture of *Staphylococcus aureus* in 30 minutes at 20° C. whereas it required 150 minutes to sterilize the same culture at 10° C.

The disinfecting method may succeed or fail depending on the **nature of the organisms to be killed**. Bacteria vary in their susceptibility to disinfectants not only from species to species, but also among the strains of one species. An organism may be highly susceptible to one disinfectant and quite resistant to another. A method which destroys the gonococcus may be ineffective against the typhoid bacillus under identical conditions. The high lipid content of the tubercle bacillus is probably responsible for its high resistance to many disinfectants. Bacterial spores are remarkably resistant to chemical agents and considerable exposure to intense heat is recommended as the only practical way to assure their destruction.

In actual practice disinfection is directed chiefly against pathogenic bacteria in body secretions, excreta and exudates, on contaminated objects and in water, milk and other foods. The **nature of the material to be disinfected** greatly influences the action of a disinfectant. The presence of coagulable organic matter protects bacteria from the effects of heat and chemical agents. It is more difficult for a disinfecting agent to reach bacteria imbedded in relatively dry viscous material than those suspended in a liquid. The moisture content, pH, the presence of electrolytes and other properties of the substance surrounding the bacteria may affect the outcome of the disinfecting method.

Many circumstances govern the choice of an appropriate disinfectant. The properties of the bactericide, the kind of microorganism and the nature of the material to be disinfected are the chief considerations. The toxicity of most powerful bactericides precludes their application to living tissues. Objectionable odor, color and corrosive properties that ruin fabrics and metals may limit their usefulness outside the body. Whether a substance is soluble in water, is able to penetrate through tissues or nonliving organic matter, is bactericidal in high dilutions, is slow or rapid in its action, is chemically stable and is not prohibitive in cost are among the factors which determine its suitability as a disinfectant.

### EVALUATION OF DISINFECTANTS

In order to evaluate the many products placed on the market, often advertised with exaggerated claims regarding their bactericidal power, a number of methods have been devised for testing disinfectants. Chief among these is a



procedure that determines the bactericidal strength of the disinfectant as compared with that of phenol, or the phenol coefficient. The phenol coefficient is a figure that expresses the disinfecting strength of a substance in terms of that of pure phenol. An official method for this determination is prescribed by the United States Food and Drug Administration (F.D.A. method). Under the standard conditions of this procedure the test organism is a 22- to 26-hour culture of strain of *S. typhosa* which has a known resistance to phenol. A satisfactory culture is one that is killed by a 1:90 dilution of phenol in 10 minutes but not in 5 minutes and that survives exposure to 1:100 phenol for 10 minutes or longer. Several dilutions of the disinfectant in question are tested to determine the best dilution (least amount) of this substance which will kill the same test organism in 10 minutes but not in 5 minutes. The phenol coefficient is then calculated by dividing the effective dilution of the test substance by the dilution of phenol which also accomplishes sterilization of the culture in the same time. One-tenth of a milliliter (0.5 ml.) of the test culture is added to each dilution of phenol and of the disinfectant being tested. At intervals of 5, 10 and 15 minutes a standard (4 mm.) loopful of the culture-bactericide mixture is removed from each dilution and inoculated into a tube of nutrient broth. After incubation the broth tubes are examined for the presence or absence of growth. In the following results of a phenol coefficient determination growth (+) means survival and no growth (—) the killing of the test organism after exposure to the given dilution of the bactericide in the stated period of time.

## DISINFECTANT X

## TIME OF EXPOSURE:

DILUTION	5 MIN.	10 MIN.	15 MIN.
1:175	—	—	—
1:200	+	—	—
1:225	+	—	—
1:250	+	+	—
1:275	+	+	+

## PHENOL

## TIME OF EXPOSURE:

DILUTION	5 MIN.	10 MIN.	15 MIN.
1:90	+	—	—
1:100	+	+	+

$$\text{Phenol coefficient} = \frac{225}{90} = 2.5$$

Therefore, under the conditions of the test, disinfectant X has two and one-half times the disinfecting strength of phenol. In the same test a disinfectant with a phenol coefficient of one is equal to phenol, and one with a phenol coefficient less than one is less effective than phenol as a bactericide against *S. typhosa*. The typhoid bacillus is the test organism when evaluating disinfectants suitable

for killing enteric bacteria in excreta and other materials. *Staphylococcus aureus* is substituted for *S. typhosa* when testing substances to be used on wounds, and other body regions, and since staphylococci are more resistant to phenol than the typhoid bacillus, the phenol coefficient will change accordingly. The test may also be run in the presence of organic matter by adding a known amount of feces, yeast or serum to the suspension of the test organism.

There are a number of objections to the phenol coefficient as a measure of practical bactericidal efficiency. It does not take into consideration such factors as the influence of temperature other than that maintained in the standard procedure, the toxicity of the disinfectant and how it acts in the presence of varying amounts and kinds of organic matter, nor does it necessarily determine the optimal disinfecting concentration. Furthermore, the phenol coefficient method is not applicable to every type of disinfectant. It is suitable only for water-soluble substances and other methods must be used to evaluate bactericidal ointments, salves, powders, oils, gases and similar preparations.

A **filter paper method** has been devised for testing substances which are insoluble or are to be used undiluted. Squares of sterile filter paper are wet with a broth culture of the test organism and then exposed to the test substance. At the end of 5, 10 and 15 minutes, or at any given time interval, a paper square is removed to a tube of nutrient broth and subcultured to determine the bactericidal or bacteriostatic action of the test substance. In testing fumigants and oils the contaminated filter paper squares are dried before exposure.

An **agar plate method** may be used to test the bacteriostatic power of pastes, powders and preparations designed for application to the body surfaces. In the standard procedure, cooled melted agar is inoculated with the test organism, usually *Staphylococcus aureus*, poured into a petri plate and allowed to harden. The test substance is placed in a spot on the surface of the agar, the plate may be covered with an unglazed, porcelain top and incubated top side up. After 24 to 48 hours a clear zone around the spot of test substance indicates no growth of the organism, and the wider the clear zone the greater are the diffusion and bacteriostatic powers of the substance. If the substance is not inhibitory to the organisms grow over the entire plate, even in contact with the test spot.

The **agar cup method** is a modification of the above procedure and is planned for testing liquids. After seeded pour plates are hardened, a plug of agar 1.5 cm. in diameter is removed by means of a sterile cork borer leaving a hollow cup. The test liquid is placed in the cup and the plate is incubated. A clear zone extending from the margin of the cup indicates inhibition of the organism. To determine whether the bacteria are killed or merely inhibited in their growth, a sample of agar from the clear zone may be removed and subcultured in a nutrient broth.

When dealing with a substance which is to be used in or on the body it is important to know not only how harmful it is to bacteria, but also how toxic it is for body tissues. No standard method has been developed for this purpose, but different **toxicity tests** have been introduced to compare concentrations



bactericide required to kill pathogenic bacteria with those which are harmful to animal tissues. In one such test a number designated as the toxicity index is used as the ratio of the highest dilution of the bactericide required to prevent death of the tissues (in a tissue culture) after 48 hours to the highest dilution required to kill the test organism in 10 minutes. In other tests toxicity is measured by the minimum amount of the bactericide which is lethal for the chick embryo or the white mouse.

## 20

# SURGICAL AND MEDICAL ASEPSIS

Practical asepsis as applied to surgery, obstetrics and the management of infected individuals has the same aims and follows the same principles as asepsis in the bacteriology laboratory. In the laboratory aseptic techniques are practiced to assure the sterility of culture media, glassware and other equipment, to prevent the entrance of foreign microorganisms into specimens and cultures, to forestall the escape of organisms from these materials into the surroundings. In the same way, sterile parts of the body are protected from contact with microorganisms in **surgical asepsis** and the escape of dangerous organisms from the infected individual is cut off by the **precautions** or techniques of **medical asepsis**.

### SURGICAL ASEPSIS

**Development of Techniques.** The history of modern asepsis begins with the development of methods to permit the healing of wounds without infection or by "first intention," and to prevent infection at childbirth. Until the latter half of the nineteenth century it was considered natural for a wound, whether accidental or surgical, to become inflamed and to discharge pus. This suppuration and "putrefaction" of wounds was accepted as the normal course of events leading to healing, a course known as healing by "second intention." That microorganisms growing in the wound were the cause of the inflammation with its exudate was unknown. The exudate itself was believed to be the "poison" responsible. The first to have reason to question the cause of this suppuration was a sixteenth-century French army surgeon, Ambroïse Paré. The standard treatment of gunshot wounds at that time was the application of scalding oil which was supposed to counteract the poison in the wound. One day when the supply of oil ran out, Paré was forced to let the wounds of his men go untreated except for the application of a soothing ointment. In 1554 he published a treatise on this new and improved treatment of gunshot wounds.

Almost 300 years later Oliver Wendell Holmes, an American physician, observed that childbed or puerperal fever could be carried from one lying-in patient to another, and in a paper read before a Boston medical society in 1829 he recommended washing the hands in calcium chloride and changing clothes before attending an obstetrical case. At about the same time Semmelweis



g obstetrician in a Vienna hospital, related the high mortality in his ward to the vaginal examinations made by doctors and students who came directly from the autopsy table to the lying-in patient. In 1847 he put his idea to work by insisting on strict cleanliness, particularly the washing of hands in a solution of chloride of lime, with the result that the mortality rate in his ward dropped precipitously. The revolutionary ideas of Holmes and Semmelweis started a controversy that lasted for years, for results were the only evidence of the soundness of their ideas and the reasons for these results were unknown. In 1860 Pasteur demonstrated that fermentation and putrefaction in organic substances were due to yeasts and bacteria, and he soon pointed out that decomposition could be prevented by heating the solutions and protecting them from contact with microorganisms in the air. Impressed by the work of Pasteur, the English surgeon, Joseph Lister, wondered whether suppuration and putrefaction of wounds might not also be due to microorganisms. At that time septicæmias, "hospital gangrene," tetanus and other diseases flourished among surgical cases. Amputations were followed by death in 45 per cent or more of patients so operated. In 1865 Lister set out to **destroy microorganisms in surgical wounds** by the generous use of carbolic acid on the patient's skin, the surgeon's hands, instruments and dressings, and later in a spray over the operative field. After two years' work he published the successful results of his **septic (anti, against; sepsis, putrefaction) method**. With the work of Pasteur and Koch furnishing definite proof that microorganisms cause disease (1877), **asepsis** gradually replaced antiseptics, and in 1891 von Bergmann established the standardized aseptic ritual followed in surgery today. The techniques of modern surgery aim to **prevent the entrance of a single microorganism into the wound, i.e., to keep sterile parts of the body sterile**.

**Examples of Surgical Asepsis.** Every move in the elaborate system of asepsis as practiced in the operating room is the outgrowth of knowledge of microorganisms and common sense methods to control them. When entering or leaving regions of the body which are normally sterile they must be protected from contact with all microorganisms in order to safeguard the patient from the danger of infection. Contamination can be prevented by knowing the sources of microorganisms in the operating room and the routes by which they may reach the sterile operative field. Important sources of microorganisms are: the mouths, noses, and throats of persons in the operating room; the skin of the surgeon's hands and of others assisting at the operation; the patient's skin and any other normally contaminated region which may be involved; all articles and materials coming in contact with the sterile field; the air of the room. With these sources of contamination in mind the reasons for each of the following aseptic techniques become clear.

**Operating Room Personnel.** All who enter the operating room wear sterile gowns, caps and efficient masks which cover the mouth and nostrils. Persons suffering from respiratory infections must not enter the room because masks are no guarantee against expelling bacteria by forced exhalations such as coughing and

sneezing. Surgeons, nurses and others assisting at operations remove as many microorganisms as possible from the skin of hands and arms by a thorough "scrubbing up" with a sterile brush, green soap and warm running water. After drying on a sterile towel the hands and arms are immersed in 70 per cent alcohol or treated with another germicide to reduce further the numbers of bacteria. Recently acquired contaminants, transient microorganisms and even the normal flora on the surface of the skin are removed or destroyed by these measures. Bacteria remain deep in the pores and, therefore, sterile rubber gloves must be worn. Any lesion on the hands disqualifies a person from taking part in an operation, for rubber gloves may be punctured and are not absolute protection.

**The Patient.** The patient's skin about the site of the proposed incision is prepared by thorough cleansing and the application of a germicide. The operating field is shaved and scrubbed with tincture of green soap, using a gauze compress or a soft brush followed by scrubbing with 70 per cent alcohol. When dry the area is painted with a suitable germicide. If tincture of iodine is used it is allowed to dry and then removed with alcohol. The prepared area is protected by sterile dressings or other coverings. On the operating table the prepared area proximal to the proposed incision is covered with sterile towels and the rest of the body is draped with one or more sterile sheets. The purpose of the draping technique is to prevent unnecessary exposure of the sterile field and to prevent contact with unprepared areas of contaminated skin.

**Instruments and Other Equipment.** Dressings, gauze sponges, towels, sheets, gowns, rubber gloves, instruments, needles and other articles contacting the sterile field are sterilized by autoclaving wherever possible to be sure the most resistant forms of bacteria, the spores, are destroyed. Sharp-edged, metal instruments and certain other articles are often "sterilized" by boiling for a few minutes or by immersion in a chemical disinfectant ("cold sterilization"), but these methods are not recommended and should be avoided. Autoclaving is not without its pitfalls. Improper packing and wrapping of supplies, sublethal temperatures due to air in the steam chamber and overloading of the autoclave can result in survival of microorganisms.

**Air and Dust.** Every effort is made to reduce the numbers of microorganisms in the air of the operating room. Walls, floor and ceiling are kept dust-free by thorough cleansing with soap and water. Operating room personnel wear clean clothing under sterile gowns and shoes that have not been used outside. The number of individuals in the room is limited. Activity and talking are reduced to the minimum. In spite of such precautions potentially dangerous bacteria have been recovered from the air of operating rooms and in an attempt to remedy this break in asepsis some hospitals have installed ultraviolet radiation and/or ventilating systems to supply the room with washed filtered air.

**Asepsis in Obstetrics.** Obstetricians and obstetrical nurses practice asepsis to prevent infection of the uterus at childbirth, and the aseptic techniques followed in preparation of the patient and in the delivery room are very much like the procedures in surgery.



injections and Collection of Specimens. The administration of fluids and the withdrawal of specimens from normally sterile regions of the body are also aseptic procedures. Blood samples are withdrawn from the body and substances are introduced into the circulatory system, as in blood transfusion, in therapy and chemotherapy, by way of a superficial vein or by venipuncture. Communication with the central nervous system may be made by spinal puncture, as in the administration of a spinal anesthetic or in the collection of cerebrospinal fluid. In performing venipuncture and spinal puncture the skin is cleansed, disinfected, usually with tincture of iodine followed by alcohol, and in the case of spinal puncture the field is protected by sterile towels or sheets, the operator wears sterile rubber gloves. Withdrawal of urine from the normally sterile urinary bladder or catheterization is also an aseptic procedure. Specimens are collected in sterile containers and are protected from subsequent contamination.

### MEDICAL ASEPSIS

**Aim and Principle of Medical Asepsis.** In the care of a patient with a communicable disease certain techniques are practiced in order to prevent the spread of the infection to others and also to protect the patient from a second infection. These techniques comprise a system known as medical asepsis. Precautions against the spread of infection are based on the facts that the patient is a source of pathogenic microorganisms, that these organisms leave the source and, unless controlled, spread into the environment to be contracted by other susceptible persons. The infected patient may be pictured as a point from which lines of microbic traffic radiate. The aim of medical asepsis is to cut these lines, *i.e.*, stop the spread of the pathogens, as close to the source as possible. Aseptic techniques differ from one type of infectious disease to another, and depend primarily on (1) the location of the infectious microorganism in the patient's body, (2) the materials or agents which carry the pathogen from the patient, (3) its resistance outside the body, and (4) how and where it must enter the body of the new victim to set up another infection. Precautions exercised in the control of respiratory infections where the pathogen leaves the body in sputum and nose and throat secretions are different from those used in enteric infections such as dysentery where the dangerous microorganisms are in the stool. To protect the patient already ill with an infectious disease, hospital personnel must be constantly on guard against the possibility that another pathogen may be introduced to set up a secondary infection or cross-infection. The danger of cross-infection is particularly imminent in wards isolating patients with respiratory infections. A patient with measles who carries a pathogenic micrococcus when admitted may be responsible for a subsequent outbreak of scarlet fever in the measles ward. In the same way it is possible for a visitor, doctor or nurse to be the source of secondary infections among the patients. The responsibility for effective medical asepsis falls chiefly on the communicable disease nurse. No one set of rules can be laid down which fits every

situation, recommended techniques vary from one hospital to another and the nurse knows **why** she goes through a certain routine, she cannot be **relied upon** to safeguard her patient or to prevent the spread of disease to others, including herself. To be efficient she must have a knowledge of the nature of microorganisms, where they multiply, how pathogens are transmitted from one person to another, if they survive for an hour or months outside the body, how resistant they are to physical and chemical disinfectants. The same is true for the intelligent care of the infectious case in the home.

It should be emphasized that techniques in medical asepsis are directed against pathogenic microorganisms. The patient harboring the pathogen is **infected**, whereas the presence of pathogens on or in inanimate objects or materials is termed **contamination** and an article is said to be **contaminated**. It is common to use the term **clean** to mean freedom from pathogens or known contamination. By comparison, **sterile** refers to freedom from all living organisms. The techniques of medical asepsis are those which insure freedom from contamination. These techniques include **isolation of the infected patient**, other precautions to prevent contamination and the **disinfection of contaminated objects and materials**. Disinfection as practiced from day to day at the bedside of the active case is known as **concurrent disinfection**. The aim of concurrent disinfection is to kill the infectious microorganisms as soon as possible after they leave the body or immediately after articles have become contaminated. **Terminal disinfection**, on the other hand, is the destruction of pathogens at the termination of the disease or removal of the patient, a sort of last round-up of pathogens contaminating the room, cubicle or unit and articles used by the patient.

**Isolation of the Patient.** Infectious diseases differ in the way or ways they are transmitted and in their degree of communicability. The infected individual, therefore, is restricted in his movements and contacts with others according to the nature of the infection. In a case of malaria, a disease not communicable by direct or indirect contact with the case, the patient need only be protected against the bite of mosquitoes which spread the disease. In certain animal diseases which are transmissible to man, such as tularemia and undulant fever, isolation of the patient is not required, but precautions, including the disinfection of body wastes and exudates which contain the pathogen, are necessary. In a disease which is communicable by direct and indirect contact with an infected human being the patient is isolated and medical asepsis is observed. Again depending on the communicability of the infection, isolation may mean a screen or chalk line around the bed on an open ward and the practice of individual precautions, the proper spacing of beds in a disease unit such as a measles ward or strict segregation of the patient in a separate room or glassed-in cubicle. The patient is thus cut off from contact with others. Visitors are not allowed to enter the patient's room or they are kept a safe distance from the patient by hospital regulations. Doctors and nurses who attend the case follow techniques to prevent contact with the pathogens. Where there is no communicable disease hospital



ly infectious cases must be cared for at home according to the regulations of local health authorities.

**Quarantine.** While isolation usually refers to segregation of the active case of infectious disease, the term quarantine is reserved for the segregation of exposed susceptible individuals for a period of time equal to the longest usual incubation period of the disease to which they have been exposed. Quarantine thus protects the public from infectious spread by apparently healthy persons (or animals) who may be infected and who may later become active cases.

**Precautions to Prevent Contamination.** The destruction of pathogens in patient's wastes and on contaminated surfaces by disinfection is part of the precautions program, but just as important is the prevention of contamination by contact with the pathogens. Doctors and nurses attending the isolated patient wear cover-all gowns. Masks may be worn when caring for cases of respiratory diseases in which pathogens are expelled by coughing and sneezing. The techniques for adjusting and removing gown and mask and for handling the patient as well as contaminated articles are designed to avoid contact with pathogens. In some infections where unavoidable contacts have been made, thorough cleansing of the hands is sufficient to remove pathogens; in other cases the hands are disinfected by immersion in a germicidal solution. Certain techniques avoid contact between the hands and contaminated surfaces by the use of instruments or towels. Paper gloves are worn when dealing with highly infectious or grossly contaminated material as when dressing infected wounds. Scrupulous cleanliness of the room and of the patient prevents reinfection of the patient and the spread of pathogens in general. Dust control measures are an important part of the precautions program. The proper instruction of the patient reduces contamination.

**Examples of Concurrent Disinfection.** A few examples will serve to emphasize the need for different disinfecting measures in different diseases. There is no intention here of giving directions for the method of disinfection cited nor of commending a particular disinfecting agent. In the respiratory diseases concurrent disinfection includes the incineration of sputum cups and paper tissues used with upper respiratory discharges. Trays with dishes used by the patient are kept separate, scraps of food are removed to paper bags which are burned, silver, dishes and tray are disinfected by boiling or exposure to live steam for ten minutes. Oral thermometers are wiped with a cotton sponge wet with soap and water and returned to a jar of disinfectant such as bichloride of mercury solution. In enteric infections, for example typhoid fever, urine, feces, vomitus and bathwater are disinfected by chlorinated lime or some other chemical agent before disposal. Bedpans, urinals and other contaminated utensils are boiled or exposed to live steam. Pathogens on soiled towels, clothing and bed linen are subjected to the lethal temperature of boiling or live steam or are soaked in a germicidal solution such as 2.5 per cent cresol.

In certain diseases the only or main escape of the infectious microorganisms from the body is from skin lesions, and in such cases precautions are directed against tissue exudates, dressings and clothing. In these cases asepsis includes

wearing rubber gloves when contacting the infected area. Dressings from infected wounds, ulcers, burns and similar lesions are incinerated. Gloves and instruments which have come in contact with contaminated dressings or the infected area are washed and soaked in a disinfecting solution if the infection is not caused by a sporulating bacterium. To guarantee the destruction of spores of tetanus, gas gangrene and anthrax all contaminated materials must be subjected to a sterilizing temperature of the autoclave.

**Terminal Disinfection.** In most infectious diseases where proper precautions have been observed throughout the illness, terminal disinfection amounts to nothing more than a thorough cleansing and airing of the room and its contents. Beds are stripped and the mattress and pillows are aired and sunned for several hours. In diseases caused by more resistant organisms, bedding is exposed to direct sunlight for a longer time or autoclaved, depending on the recent infection. Wherever possible equipment including utensils and articles used by the patient are boiled or exposed to live steam. Books and other articles which are contaminated by resistant organisms and which cannot be disinfected by sunlight, moist heat or chemicals are burned, or, if the pathogen survives but a short time outside the body, they may simply be kept out of circulation for a suitable time. Before being dismissed the patient is given a bath and shampoo, dressed in clean clothing and transferred to a clean room or unit.



## 21

# CHEMOTHERAPY

Chemotherapy is the treatment of infectious disease with substances which either inhibit the growth of the pathogenic organisms in the host's body without causing injury to the host. Successful chemotherapeutic substances are selective in their action, readily combining with and damaging the protoplasm of the pathogen while having little or no affinity and toxicity for the protoplasm of the host. However, a substance may exhibit marked antimicrobial action *in vitro*, but produce no toxic effects when introduced into the animal or human body, and have no clinical value if it is not effective in attacking the agent of disease in the presence of body substance and exudates. Only *in vivo* tests can determine whether nontoxic concentrations of the agent can attack the microorganisms in the presence of tissue products, blood, serum and pus.

The selective action of chemotherapeutic agents depends on the relationship between the chemical nature of the agent and the chemistry of living cells. Just as a substance may be destructive to the cells of a certain bacterial species and harmless to human tissues, so it may be innocuous to other unrelated microorganisms. For this reason chemotherapy is now successful in combating only certain infectious diseases. In general, protozoa and bacteria are more susceptible to the known chemotherapeutic agents than are the molds, yeasts, rickettsiae and viruses. Among the pathogenic bacteria, spirochetes, cocci and certain gram-negative bacilli are more readily attacked than the gram-negative and acid-fast bacilli.

**Paul Ehrlich and 606.** The story of chemotherapy begins with the discovery and development of the aniline dyes, their application to tissue staining and, finally, the vision of a German chemist, Paul Ehrlich. In 1871 the pathologist Robert Koch demonstrated that the bacteria in a section of infected tissue stain more readily with the aniline dyes than the cells of the tissue. This fact inspired Ehrlich to investigate the possible use of these dyes as bactericidal substances in the infected body. He introduced the term chemotherapy to signify the destruction of living agents of disease within the body by chemical means. The aim of his research was to synthesize a substance which in one dose would sterilize infected tissues without injuring the infected host, *i.e.*, to find a powerful, non-toxic, internal bactericide. Up to this time effective chemotherapy was available only in certain protozoal diseases. In 1910 Ehrlich announced his success in syn-

thesizing the first effective chemotherapeutic agent against a bacterium. This was an arsenic-containing aniline compound designated first as 606 (being the compound synthesized in the series numbered 600) or **salvarsan** and now known officially as **arsphenamine**. It destroyed the spirochete of syphilis in the blood but not by a single dose and not without some danger to the patient. Ehrlich improved his product by making the less toxic neosalvarsan (neoarsphenamine).

**The Sulfonamide Drugs.** Following this discovery thirty years elapsed before another satisfactory chemotherapeutic agent against bacteria was introduced. Then in 1935 Domagk announced that **prontosil**, a red dye technologically named sulfonamide-crysoidin, would cure streptococcus infections. Later in the same year French scientists disclosed that the colorless **sulfanilamide** portion of prontosil was the active ingredient, and that it was as effective as the later conjugated molecule. Soon other forms of sulfonamide drugs were developed, and it became evident that a number of bacteria, particularly pneumococci, gonococci and meningococci, as well as streptococci, were subject to their action. Then followed the synthesis of sulfonamide derivatives, such as sulfadiazine, sulfamerazine, and sulfamethazine, which are potent antibacterial agents and less toxic than sulfanilamide. Poorly absorbed sulfonamide compounds—for example, sulfaguanidine, sulfasuxidine and sulfathalidine—are now available for treating certain intestinal infections.

The sulfonamides are not bactericidal *in vivo*, but they inhibit multiplication of the bacteria and are, therefore, bacteriostatic. Sulfonamide therapy is most effective in the early stages of infection to check the growth and spread of bacteria, and it must be continued until the defense mechanisms of the body have time to eliminate them. The action of the sulfonamides has been explained as one of **competitive inhibition** in which the sulfonamide molecules become fixed to the bacterial cell in place of the molecules of a structurally similar, essential growth substance. In this way they interfere with the normal functioning of certain enzyme systems. This explanation is supported by the fact that the vitamin nicotinic acid blocks the bacteriostatic action of sulfapyridine, and an excess of the growth factor, *para*-aminobenzoic acid, counteracts the inhibitory effect of sulfanilamide and a wide variety of sulfonamide derivatives. Sulfonamide-sensitive organisms must synthesize the essential growth factor, folic acid, from *para*-aminobenzoic acid. Sulfonamide molecules prevent this utilization of *para*-aminobenzoic acid. In culturing specimens taken from patients receiving sulfonamide therapy, 5 mg. of *para*-aminobenzoic acid are added to each 100 ml. of medium to allow growth of streptococci or other sulfonamide-susceptible bacteria which may be present.

Sulfonamide therapy is not a "cure all" because only certain bacteria are susceptible to the action of these drugs. For this reason the identity of the infectious agent should be determined before therapy is initiated. Furthermore, susceptible organisms may give rise to resistant variants during therapy, particularly when inadequate dosage is administered. The problem presented by the development of sulfonamide-fast strains of an originally susceptible bacterium



expressed in the following statement: "Until recently sulfonamide compounds were effective against gonorrhea and gonorrheal arthritis in a high percentage of cases, but the sulfonamide-susceptible strains of gonococci have now largely been killed off in the United States, leaving mostly the sulfonamide-resistant strains to be combated." \*

Good results have been obtained in the treatment of leprosy with three sulfonamide-related drugs, namely the sulfone compounds, promin, diasone and thiothiazole. Experimental tuberculosis in animals treated with these compounds gives results which indicate the advisability of evaluating the efficacy of sulfone therapy in human tuberculosis. To date there is no successful sulfonamide chemotherapy available for most of the virus diseases, and the mycotic and rickettsial infections do not respond to these drugs. *Para*-aminobenzoic acid has proved to be an effective agent in the treatment of the rickettsial diseases, typhus fever, tsutsugamushi (scrub typhus) and Rocky Mountain spotted fever.

**Antibiotic Therapy.** A new type of chemotherapeutic agent was destined to augment and in many cases replace those made by the chemists. These new products are naturally occurring substances of microbial origin which are antagonistic to other living cells. The term **antibiotic** (literally, against life) now is restricted to antimicrobial substances produced by or derived from living bacteria, yeasts, molds and other plants. One of these substances, penicillin, most closely approaches the ideal chemotherapeutic agent. A recent article on the treatment of infectious diseases begins with these statements: "Needless to say most of what is new revolves about antibiotic agents. So rapid has progress been in the use of sulfonamide compounds are now regarded as old fashioned in the treatment of certain diseases and immune serums are nearly obsolete." †

**Penicillin. Discovery of Penicillin.** In 1929 Dr. Alexander Fleming, an English bacteriologist, noted that there was no growth of *Staphylococcus aureus* at a considerable distance around a colony of mold which had accidentally contaminated his plate culture. Antagonism between microorganisms had been observed before, but Fleming's observation was the first to lead to the discovery of a new and powerful weapon against infectious disease. The mold, which proved to be *Penicillium notatum*, produced a high concentration of an antibacterial substance in nutrient broth culture, and a filtrate of the culture was more toxic for laboratory animals than sterile nutrient broth. Fleming named the substance "penicillin" and suggested its possible usefulness in the treatment of infectious diseases. Here the matter rested until 1937 when a group of Oxford scientists initiated a program to perfect methods for isolating the active ingredient in Fleming's crude penicillin and to evaluate its clinical worth. Today the use of penicillin in the treatment of bacterial infections is without precedent, and it is now produced on a large scale in a purified form by chemical and pharmaceutical manufacturers.

Adaptation for survival in the struggle for existence among microorganisms

\* P. S. Hench: *J.A.M.A.*, 132:974, 1946.

† H. A. Reimann, *J.A.M.A.*, 132:969, 1946.

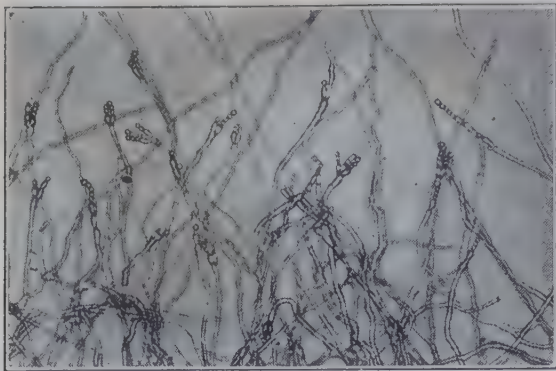


Fig. 130. Photomicrograph of *Penicillium notatum*. ( $\times 460$ .) (Courtesy of Abbott Laboratories.)

includes the secretion of antibiotic substances which interfere with the metabolism and thus repress the growth of competing or "enemy" organisms. The discovery of penicillin stimulated search for other antagonistic substances of microbic origin to inhibit the growth of pathogenic bacteria. Many such products from molds, soil bacteria and higher plants have been discovered, but so far none has equalled penicillin in its antibiotic activity and its lack of toxicity to the body. It is fairly safe to predict

however, that penicillin will not remain forever unrivaled.

**Production of Penicillin.** Strains of the penicillin-producing mold, generally *Penicillium notatum*, are grown in fluid cultures under carefully controlled conditions designed to stimulate maximum penicillin production. The mold can be grown in an aqueous solution of inorganic salts and sugar, but penicillin yield is markedly increased by the addition of corn-steep liquor to the culture medium. The proper reaction ( $pH$  7 to slightly acid) and mineral content of the medium, an incubation temperature close to  $24^{\circ} C$  and adequate oxygen supply are important factors in penicillin production. Strict asepsis must be practiced throughout production to prevent contamination of the cultures.

**Surface cultures** of the mold may be grown in shallow layers of the nutrient solution in Erlenmeyer flasks or similar, broad, glass vessels which allow for aeration of the culture, or the **submerged culture** method which is suitable for large-scale production of penicillin may be used. In the latter method a strain of *P. notatum* adapted to subsurface growth is cultured in large tanks or vats in which there is constant aeration and agitation. As the blue-green mold matures, the culture medium becomes yellow owing to a soluble pigment, crysogenin, which is not an antibiotic but is an index of penicillin production since its development parallels that of penicillin. Maximum penicillin production is reached



Fig. 131. Surface culture of *Penicillium notatum*. (From Kolmer *Penicillin Therapy*, 2nd ed., D. Appleton-Century Co.)



about one week in surface cultures and in 2 or 3 days in submerged cultures. Samples taken from the cultures are assayed at intervals to determine when the highest penicillin concentration is attained, and at this time the mold is separated from the fluid of the culture by filtration. Penicillin is then extracted from the filtrate and purified. In the filtrate penicillin occurs as an acid which readily forms various salts, such as potassium, calcium and sodium penicillin, and some of these salts have been isolated in pure crystalline form. The penicillin of culture filtrates is not a single substance but consists of several closely related antibiotic substances which have been designated penicillin F, G, X and K. The product available through large-scale commercial production is a crystalline form of penicillin G (benzyl penicillin). Other antibacterial substances not suitable for therapeutic purposes have been isolated from *P. notatum* cultures and named notatin, penatin and penicillin B by various workers. It is possible that these three factors are identical. The final purified penicillin product is tested for sterility, the absence of pyrogens (fever-producing substances) and antibacterial power.

### Detection and Standardization

**Penicillin.** Microbiological tests are used to measure the *in vitro* bacteriostatic effect of penicillin on susceptible bacteria, usually a penicillin-sensitive strain of *Staphylococcus aureus* or *Bacillus*

*subtilis*, and also to measure the level of penicillin in the body fluids of patients. The unit of penicillin is an arbitrary measure of bacteriostatic strength which serves as a standard of reference or a "yardstick" with which other penicillin preparations and penicillin-containing materials can be compared. The Oxford unit is the equivalent of a standard penicillin solution which inhibits the growth of *Staph. aureus* in a zone about 25 mm. in diameter when tested by the cylinder plate method (to be described shortly). Chemically pure crystalline penicillin G with a potency of 1650 penicillin units per milligram is now available from manufacturers as a reference standard.

Several methods have been described to assay penicillin in commercial preparations, blood, serum, spinal fluid, exudates and other body fluids. These include the Oxford cup or cylinder plate method, the filter paper disc method, and the serial dilution method, the last two being in common use today. The Oxford cup method is a modification of the agar cup method (see



Fig. 132. Oxford cup assay method. Antibiotic potency is indicated by inhibition of bacterial growth (clear zones) around the cylindrical cups which contain varying concentrations of penicillin. The light area beyond the inhibition zones represents growth of the test organism. (Courtesy of Abbott Laboratories.)

page 230), and is suitable for most fluids except blood. In this method sterile glass, porcelain or stainless steel cylinders of uniform size are warmed before they are placed on the surface of agar pour plates which have been seeded with a penicillin-sensitive strain of *Staph. aureus* or *B. subtilis*. In any way as many as six cylinders can be sealed to the agar at equidistant points on the plate. The cups are filled with the fluid to be tested or dilutions of this fluid and the plates are then incubated. At the same time dilutions of a standard solution containing a known number of penicillin units per milliliter are tested on another plate. The penicillin diffuses out into the agar and inhibits the growth

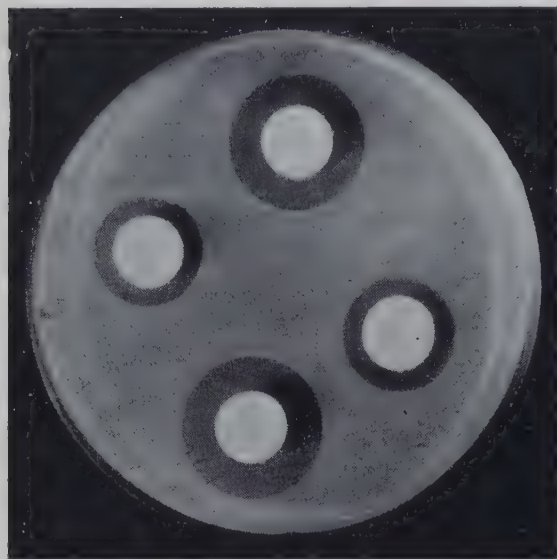


Fig. 133. Paper disc assay method. (Courtesy of Abbott Laboratories.)

of the test bacterium in a zone around the cylinders. By comparing the diameters of the zones of inhibition produced by the standard and the test fluid, the number of units of penicillin per milliliter in the latter can be calculated. A modification of the Oxford cup method involves the use of filter paper discs instead of cylinders. Standard size discs of the absorbent paper are placed on the surface of previously inoculated agar plates. A given amount of penicillin solution is delivered immediately to the center of each disc. The diameter of the inhibitory zone around each disc is in direct proportion to the concentration of penicillin in the fluid tested.

The serial dilution method is often employed to measure penicillin levels in the patient's body fluids. In this tube procedure one determines the greatest dilution of the specimen which prevents growth of the test organism in a liquid medium. At the same time dilutions of a standard solution are added to another set of inoculated broths and after incubation one notes the smallest amount of standard penicillin (expressed in penicillin units) which prevent the growth of the test organism. From these figures the number of penicillin units per milliliter of the specimen can be calculated.

Various other methods, such as slide and capillary tube tests which depend on the inhibition of hemolysis when a susceptible strain of a hemolytic streptococcus is grown in a penicillin-containing blood medium, have also been devised for assaying penicillin in body fluids.

The above methods for penicillin assay, or modifications thereof, are also used in tests for penicillin sensitivity of organisms. In this case, the bacterium of unknown susceptibility is subjected to the action of known and varying amounts of penicillin.

**Action of Penicillin.** The important human pathogens which are most susceptible to the action of penicillin include the *beta* hemolytic streptococci



cumococci, meningococci, gonococci, most strains of staphylococci, clostridia, diphtheria bacillus, the anthrax bacillus and spirochete of syphilis. It will be noted that except for the meningococcus and gonococcus all the true bacteria in this list are gram-positive. Penicillin is moderately active against certain other bacteria, but in general the gram-negative bacilli and, with rare exceptions, the nonbacterial agents of disease are insusceptible. Different strains of a susceptible species may vary considerably in degree of sensitivity to the action of penicillin, and completely resistant strains of some usually susceptible species are also encountered. A strain originally sensitive to penicillin may become resistant either *in vitro* or *in vivo*, although the development of a resistant strain of the pathogen in the patient receiving penicillin therapy occurs far less frequently than in the case of sulfonamide therapy. Whenever possible before starting penicillin treatment the organism causing the disease should be isolated from the patient and grown in the presence of penicillin to determine whether one is dealing with a penicillin-sensitive or resistant strain.

Penicillin may kill large numbers of bacteria in a culture, but in concentrations administered to patients its action is probably bactericidal only for certain organisms and bacteriostatic for the rest. Just how penicillin exerts its antibacterial action is unknown, but the evidence points to some interference with the cell's normal anabolic, protein-synthesizing processes. Penicillin is most active against young, actively metabolizing cells. Not much is known about its effect on the toxic products of susceptible bacteria, but reports suggest that penicillin does not detoxify preformed toxins, but may inhibit the production of certain toxins. Penicillin is practically nontoxic. A small percentage of patients, however, show sensitivity reactions, *i.e.*, individual allergic responses to the drug. Most of the toxicity of early preparations was due to the presence of impurities, and since the initiation of therapy with the pure salts of penicillin, generally sodium penicillin, the untoward reactions in patients have been greatly reduced.

**Penicillinase and Other Penicillin Inhibitors.** Many bacteria, including species of *Streptomyces* and spore-forming bacilli of the soil, *Esch. coli*, *Mycobacterium tuberculosis* and some strains of *Staph. aureus* and dysentery bacilli, produce a substance known as **penicillinase** which inactivates penicillin. Production of penicillinase does not necessarily mean that an organism is completely resistant, but penicillinase-producing strains of species which are ordinarily highly susceptible show increased *in vitro* resistance to penicillin action. However, the *in vivo* development of a resistant strain need not be accompanied by acquired penicillinase production. In mixed infections the presence of a penicillinase-producing organism will diminish the action of penicillin on susceptible bacteria, and its bacteriostatic effect is undoubtedly reduced in the intestine by penicillinase-producing bacteria of the normal flora. Penicillinase can be isolated from bacterial cultures and used as an inhibitor of penicillin in laboratory procedures. Other substances, such as cysteine hydrochloride, clarysase and taka-amylase, also inhibit the action of penicillin. Penicillinase, or one of these inhibitors, is added to the culture media in tests to determine the sterility of

commercial penicillin preparations and to blood cultures or cultures of specimens taken from patients receiving penicillin therapy. In this way penicillin-sensitive bacteria which otherwise would be suppressed by the penicillin in culture are allowed to grow.

**Streptomycin.** In 1944 the discovery of streptomycin, an antibiotic produced by strains of a soil Actinomycetes, *Streptomyces griseus*, was announced by Schatz, Bugie and Waksman. In many ways the properties of streptomycin

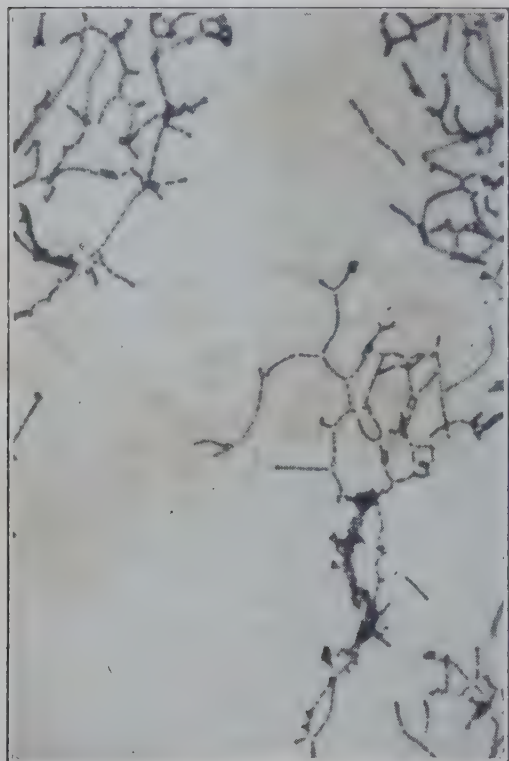


Fig. 134. *Streptomyces bikiniensis*, a newly described, streptomycin-producing actinomycete, isolated from a Bikini soil. ( $\times 545$ .) (Johnstone and Waksman: *J. Bact.* 55:317, 1948.)

proved to be very similar to those of penicillin but, by *in vitro* tests, streptomycin was shown to be active against gram-negative and acid-fast bacteria as well as against certain gram-positive bacteria. Compared to penicillin it is generally more effective against gram-negative bacilli and less active against gram-positive bacteria, with the exception of the tubercle bacillus. Obligate anaerobic bacteria appear to be susceptible to streptomycin action. Among the streptomycin-susceptible, gram-negative bacilli are members of the enteric group, *Esch. coli*, *S. typhosa*, dysentery bacilli and *Proteus vulgaris*, as well as *Hemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Pasteurella tularensis*.

Clinical trials have shown that streptomycin is of greatest value in the treatment of tularemia, and that it is also effective in meningitis, septicemia and urinary tract infections due to certain gram-negative bacilli. Results of streptomycin therapy in the enteric infections caused by *Salmonella*

and dysentery bacilli are inconclusive, and its value in typhoid fever is doubtful. The promising results obtained in studies of the effect of streptomycin on checking the course of experimental tuberculosis in animals have encouraged its use in human tuberculosis. To date, streptomycin and its derivative, dihydrostreptomycin, have proved of value in the treatment of certain forms of clinical tuberculosis.

Susceptible bacteria acquire resistance to streptomycin much more rapidly than to penicillin. It is advisable to determine whether the cause of the disease is a streptomycin-sensitive or resistant organism before instituting therapy, and to check its effect on the organism if in the course of therapy the patient fails to respond. The administration of adequate doses at the start of treatment is even more important in streptomycin than in penicillin therapy in order to prevent the development of a resistant strain.



The mechanism by which streptomycin acts against bacteria is unknown, but appears to be an interference with normal anabolic processes resulting in the destruction of the bacterium or prevention of its growth and multiplication as in the case of penicillin. No streptomycin inhibitor of bacterial origin similar to penicillinase has been reported, but streptomycin is inactivated by a number of reducing agents. Cysteine is used to counteract its antibacterial effect in sterility tests of streptomycin preparations intended for therapeutic purposes and in cultures of blood and other specimens taken from patients receiving streptomycin.

Streptomycin as it occurs in filtrates of *S. griseus* cultures is an organic nitrogenous base. After isolation of this substance from filtrates various crystalline products have been prepared, including streptomycin hydrochloride and streptomycin sulfate. The unit of streptomycin is now expressed as the amount equivalent (in bacteriostatic effect on *Esch. coli*) to 1 microgram (0.001 mg.) of pure streptomycin base. The amount of streptomycin in body fluids is determined by microbiological methods which are modifications of the Oxford disk, serial dilution and slide tests used in assaying penicillin. Various test organisms, including susceptible strains of *Staph. aureus* and *B. subtilis*, are used in different laboratories for streptomycin assay.

The toxicity of streptomycin for man and animals is low, but it is sufficient to limit this drug as an ideal chemotherapeutic agent. Untoward reactions do occur, and these reactions depend largely on the route of administration, dosage and duration of therapy. Prolonged streptomycin therapy not uncommonly results in inner ear disturbances and, by damage to the eighth cranial nerve, occasionally deafness. Attempts are being made to overcome the limitations imposed on streptomycin by its toxicity. Recent clinical experience in the treatment of tuberculosis with a new form of this antibiotic, dihydrostreptomycin, indicates that it is less toxic than streptomycin.

**Tyrothricin.** In 1939 Dubos reported that a soil organism, *Bacillus brevis*, produced an antibiotic substance that was highly bactericidal for gram-positive bacteria, particularly *beta* hemolytic streptococci, pneumococci and most strains of staphylococci, and for this reason he named the substance **gramicidin**. Further chemical studies revealed that a second antibacterial substance was associated with gramicidin and this substance, which is more effective against gram-negative bacteria than gramicidin, is now named **tyrocidine**. Gramicidin and tyrocidine seem to have a detergent action on susceptible bacteria which injures and lyses the cells. The mixture of gramicidin and tyrocidine is known as **tyrothricin** and it is this preparation that is supplied for therapeutic purposes.

Tyrothricin causes hemolysis of red blood cells, is toxic for living cells and cannot be introduced parenterally, *i.e.*, into the body tissues. This limits its use chemotherapeutically to infections that are accessible to topical applications such as wet packs, irrigations, sprays and ointments. However, it may be introduced into regions of local infection such as the urinary bladder and body cavities where it will remain in contact with the infecting agent. Tyrothricin has been found

to be effective in combating gram-positive bacteria in wounds, burns, ulcers infected sinuses. The use of gramicidin alone has been recommended since it is less toxic than the tyrocidine fraction of tyrothricin.

**Other Antibiotics.** Many substances have been recovered from bacteria, molds and higher plants which have powerful *in vitro* action against bacteria but are unsuitable for clinical use because they are too toxic or they lose their activity in the presence of body fluids and exudates (see Table 8, page 23). Such substances include streptothricin (produced by *Streptomyces lavendulae*), actinomycin (*Streptomyces antibioticus*), clavacin (*Aspergillus clavatus*), aspergillilic acid (*Aspergillus flavus*) and gliotoxin (*Gliocladium fimbriatum* and other molds). Several recently isolated antibiotics give promise as valuable chemotherapeutic agents. Among these are subtilin and bacitracin, products of strains of *Bacillus subtilis*; the closely related, if not identical substances, polymyxin (*Bacillus polymyxa*) and aerosporin (*Bacillus aerosporus*); chloromycetin and aureomycin isolated from different species of *Streptomyces*. Certain of these new antibiotics have had insufficient clinical trial for their proper evaluation. Others, notably bacitracin, chloromycetin and aureomycin, offer great hope as chemotherapeutic agents which may be successful where previously known agents have failed. Bacitracin, which has practically the same antibacterial range as penicillin, is effective in the presence of penicillinase-producing, penicillin-resistant strains. Chloromycetin, which attacks many of the streptomycin-sensitive, gram-negative organisms, has the great advantage of being active also against rickettsiae. Chloromycetin therapy has given impressive results in scrub typhus and typhoid fever. Aureomycin acts against gram-positive cocci, certain gram-negative bacteria including *Brucella*, rickettsiae and certain viruses. Clinical trial of neomycin, newly discovered *Streptomyces*-produced antibiotic which is active against a variety of bacteria, especially mycobacteria, including streptomycin-resistant strains of the tubercle bacillus, will be followed with interest.

\* Waksman, S. A., and Lechevalier, H. A., *Science*, **109**:305, 1949.



# Microorganisms and Disease

## 22

### PARASITISM AND PATHOGENICITY

Early theories variously related disease to possession by evil spirits, punishment for sin and unidentified evil influences or miasmas. Foul air, such as that from swamps, was often thought to cause disease, an idea that survives today in the name of the disease malaria, which literally means bad air. Another theory, that of Hippocrates, related sickness to an imbalance of four body humors, the yellow and black bile, the phlegm and the blood, a proper balance of which was believed to be present in health. With the development of modern medicine, these theories have gradually been replaced by views based on scientific knowledge. Thus, the work of Pasteur, Koch and others demonstrated the role of living organisms in disease, and established the **germ theory**. Today, disease production by microorganisms is clearly recognized and forms the basis of many practical methods of prevention and treatment. However, the relationship of these infectious diseases to a far broader condition known as parasitism must be further recognized.

The higher animals, including man, are well populated throughout life with countless numbers of smaller living creatures of which some are beneficial, others are not ordinarily harmful, and only some regularly cause disease. Although physicians and nurses are primarily concerned with this last group of harmful microorganisms, it is important to understand the principles of parasitism in order to understand the nature of infectious disease.

#### PARASITISM

Parasites are organisms which exist entirely or partially within or upon other organisms, so that the parasite is in some degree dependent upon the host for the maintenance of life. Parasites are recognized among the fungi, bacteria, *Klebsiellae*, viruses, worms, insects, ticks and mites. These parasites become adapted to their existence upon or within the host and differ in many respects from their saprophytic relatives. In general, they are more exacting in their growth requirements than are the saprophytes. The parasitic bacteria, for example, often require enriched media for cultivation in the laboratory, and the *Klebsiellae* and viruses multiply only in the presence of living cells. Parasites grow most luxuriantly at temperatures near the body temperature of the host. With the animal parasites and the smaller microorganisms multiply rapidly, the

offspring of many parasites being several times the number produced by related saprophytes. Among the bacteria, toxins and capsules are produced more frequently by parasites than saprophytes.

Various degrees of association or dependency of parasites and their hosts are commonly recognized. The organisms that live on the external surface of the body are termed **ectoparasites** (*ecto*, without). Among the ectoparasites are the body louse and the flea, which, except for short periods, live in close association with the animal body. Some ectoparasites, such as the mosquitoes, ticks, and many flies, are in general free-living, and visit the host only periodically or temporarily. The **endoparasites** (*endo*, within) live within the body, either within the deep tissues or in the gastro-intestinal, respiratory or genito-urinary tracts. Many endoparasites live almost continuously within the body and are unable to survive for relatively short periods of time in the external environment. Others, such as the hookworms, are in part free-living. As previously indicated, the parasites maintain life at the expense of the host and are, therefore, benefited by the association. The host on the other hand is in only a few instances dependent upon its parasites. This state of mutual dependency of host and parasite is known as **mutualism**, and may be exemplified by the case of the termites and some of their intestinal parasites, which aid in the digestion of foodstuffs. **Symbiosis**, or dependency of two species to the degree that life apart is impossible, is seldom encountered. In another type of relationship, known as **commensalism**, the host may be uninjured by the parasite but is not ordinarily benefited by the association. The organisms of the normal flora of the animal body may in their usual locations be included among the commensals. If introduced into other regions, however, these organisms may produce injury and disease in the manner of opportunists. *Bacterium coli* (*Esch. coli*), for example, normally produces no injury when confined to the intestinal tract, but in certain other regions of the body it may give rise to severe infections. The true pathogens differ from the opportunists in that they characteristically produce disease by infection in the normally susceptible host. For example, infection of man with the bacteria of typhoid fever and diphtheria or the malarial parasites commonly results in disease.

The parasites, and particularly pathogens, are limited in their ability to infect different host species. The parasites of plants, thus, are different from those of animals; and in general the disease agents of the cold-blooded animals do not invade the warm-blooded mammals. Indeed, some parasites are strictly limited to one, or a very few, closely related hosts. In general, however, it may be said that the majority of parasites, given the opportunity, may infect several different host species. Thus, certain birds harbor parasites that may infect mammals as do a number of different mammalian species, such as dogs, cattle, rabbits and a number of the rodents.

In order to survive, parasites must be transmitted from one host to another. This transfer may occur by any of a number of different mechanisms, such as in water, air, milk, and food or by living vectors, such as insects. The means



Transmission is a fundamental part of the concept of parasitism and is characteristic for each parasite. Parasites are able to survive for long periods of time within the host population by transfer from one individual to another.

Parasitism is not solely a one-sided activity. Indeed, the host is provided with many natural mechanisms by which it combats invasion by parasites and, furthermore, the host may develop an increased resistance or immunity as the result of infection with specific microorganisms.

Because it represents a reaction on the part of both the host and the parasite, parasitism is conveniently looked upon as a conflict, the balance or equilibrium of which may be tipped in favor of either contestant. Disease, thus, may be thought of as a conflict between host and parasite in which the former is injured.

**Bacteria Normally Present on the Body.** The microorganisms that characteristically parasitize the normal body are acquired during and after birth. Throughout life an abundance of organisms live on the surface of the body, in the nostrils, the openings of hair follicles, the tonsillar crypts and about the teeth. These organisms are ordinarily neither harmful nor beneficial to the host. At times, however, they have been suggested as the causative agents of certain poisonings (particularly the now unrecognized, so-called "ptomaine" poisoning; food poisoning, p. 510) and have been found to cause disease elsewhere in the body. The beneficial effect of certain normal parasites has already been mentioned in the case of intestinal parasites of the termite, and may be exemplified further by the synthesis of vitamins utilized by man by the parasitic bacteria in the intestinal tract.

The composition of the normal flora is in part determined by the chance contact of the individual with microorganisms, and in part by conditions that promote or inhibit growth of particular organisms in different locations within the body. Such factors as the diet, the acidity of secretions, such as the gastric juice, and the removal of organisms from the body in secretions and excretions are of importance.

The skin is continuously subject to contamination with a variety of organisms, relatively few of which remain long viable on the clean healthy surface. Staphylococci are the most common organisms found on the skin; sarcinae, coccobacilli, corynebacteria, bacilli, streptococci, *Bact. coli* (*Esch. coli*) and *Proteus* organisms are also frequently present.

The conjunctivae contain relatively few organisms, although *Staphylococcus aureus* and the pseudodiphtheria bacterium, *Corynebacterium xerosis*, are found in small numbers.

The flora of the respiratory tract differs in different regions. Staphylococci, coccobacilli and less commonly alpha-type streptococci are isolated from the nose. The paranasal sinuses, the lower trachea and bronchi are normally sterile. The mouth and pharynx contain many microorganisms. Smear and culture techniques usually demonstrate staphylococci, alpha-type streptococci, coccobacilli, neisseriae, such as *N. catarrhalis*, *N. flava* and *N. pharyngis sicca*.

micrococci and pseudodiphtheria bacilli. Less frequently saprophytic sporulating rods, such as *Bacillus subtilis*, hemolytic streptococci, pneumococci, and *Streptococcus pneumoniae* (Friedländer's bacillus) are isolated. In addition, numerous spirochetes, spirilla, fusiform bacteria and actinomycetes are found about the teeth and in the tonsillar crypts. Spirochetes of the mouth and pharynx include *Borrelia (Treponema) vincenti*, which is associated with trench mouth, *T. microdentium* and *T. microdentium*.

The normal acid-secreting stomach is relatively free of bacteria, but in the absence of acid and in obstruction, sarcinae, the Boas-Oppler bacillus (a large gram-positive rod) and numerous mouth organisms are commonly present. Bacteria are found in the upper portion of the small intestine, but increasing large numbers are encountered in the ileum and colon. In the adult a variety of organisms is present in the intestinal contents, including *Bact. coli* (*Esch. coli*), other enteric bacteria, enterococci, and anaerobic organisms. *Clostridium welchii* and *Cl. tetani* may be isolated by suitable methods.

The male genito-urinary tract is normally free of bacteria except externally where an acid-fast organism, *Mycobacterium smegmatis*, is characteristic. The vaginal flora of the adult is limited in part by acidity and hormonal changes. A large lactobacillus known as the bacillus of Döderlein is characteristic of the adult flora, although staphylococci and diphtheroid rods are commonly cultured. During the first few weeks of life the flora is similar to that of the adult whereas during childhood and after the menopause a greater variety of organisms, including staphylococci, nonhemolytic streptococci, anaerobic bacteria, fungi, diphtheroid rods, and *Bact. coli* (*Esch. coli*) may be cultured.

### DISEASE PRODUCTION BY MICROORGANISMS

The ability of microorganisms to produce disease has been found to be dependent largely upon four conditions: the virulence of the microorganism, the number of microorganisms which enter the host, the location of the parasite within the body and the susceptibility of the host to the infection. These factors will be discussed separately in the succeeding pages, but it should be understood that disease results from their interaction and that in practice it may be difficult or impossible to separate their several roles in a given disease. The infection with a large number of moderately virulent organisms may affect the host as severely as a small number of more virulent organisms. Similarly a highly susceptible host may be severely affected by an infection of little consequence to a more resistant individual. Most organisms can enter and establish infection only if introduced by a suitable route or portal of entry into tissues in which they are able to live and multiply. For example, the diphtheria bacillus is unable to penetrate the skin but readily infects the mucous membrane of the nose and throat and remains localized in this site; the bacterium of typhoid fever, on the other hand, enters through the gastro-intestinal tract and invades the deep tissues and blood.



**Proof that Microorganisms Cause Disease.** Before a microorganism may be said to cause disease, proof must be obtained of its role in the production of injury. Early in the history of bacteriology a set of rules, known as Koch's postulates, was formed to guide investigators in their search for disease agents. Fulfilment of Koch's postulates provides excellent proof that a suspected organism is actually responsible for the disease. These postulates may be stated as follows:

1. The organism must be observed in every case of the disease.
2. The organism must be isolated and grown in pure culture in the laboratory.
3. The disease must be reproduced in a susceptible animal by inoculation of the pure culture.
4. The organism must be observed in and isolated from the diseased animal.

The postulates have been fulfilled in the case of many diseases, for example tuberculosis, anthrax and tetanus. Unfortunately, it is not always possible to obtain such direct and conclusive evidence against the microorganism. This is particularly true in those diseases due to the viruses, for these agents can neither be isolated on culture media nor can they be seen by the usual methods. The viruses, however, are known only as agents of disease. Their presence in diseased tissues may be consistently demonstrated by inoculation of laboratory animals, and additional evidence against them is available in that man and animals convalescent from infection are generally immune or resistant to later infection with the same agent. The blood of these immune individuals specifically protects other susceptible animals against infection. Specific immunity is developed against other agents of disease as well as against the viruses and in all cases provides strong evidence against the microorganism.

Before a microorganism may be established as the cause of a disease, all of the incriminating evidence against it must be carefully weighed. If the evidence is reasonably complete, there is little doubt of the role of the suspected agent. One should continue only to suspect the organism if the proof of its role is incomplete or the evidence is conflicting. In such cases additional investigation has not infrequently shown another agent to be responsible for the symptoms of the disease.

**The Portal of Entry.** Microorganisms may enter any region of the body in direct or indirect contact with the external environment. The pathogens invade through wounds, abrasions of the skin and the mucous membranes or are introduced by biting insects. The diphtheria bacillus readily infects the mucous membranes, particularly those of the nose and throat. The organisms of typhoid fever, dysentery and cholera enter through the intestinal tract. Those causing tetanus and gas gangrene produce severe disease when introduced into wounds. The causative agents of tularemia, hookworm disease, anthrax, plague and certain other infections are able to penetrate the normally intact skin or enter through hair follicles and small injuries. The malarial parasites are introduced through the skin by infected mosquitoes. Of these possible portals of entry the mucous

membranes of the respiratory, gastro-intestinal and genito-urinary tracts the most common sites of invasion by microorganisms. The portal of entry naturally occurring disease is characteristic, so that disease agents may be highly infective by one route but ineffective if otherwise introduced.

The route by which organisms leave the body, the **portal of exit**, is likewise characteristic and is largely determined by their habitat within the body. Those which cause disease of the respiratory tract leave in the discharges of the nose and throat. Pathogens of the intestinal and genito-urinary tracts leave in the feces, urine and other discharges. Organisms that infect the skin find exit through exudates from skin lesions. Biting insects may acquire parasites directly from the blood.

**Bacterial Virulence.** Virulence has been defined as the ability of organisms to produce disease. Injury in some instances results from the spread of organisms through tissues or into the blood, *i.e.*, by tissue invasion. The ability to penetrate or invade the tissues is termed **invasive power**. Streptococci, pneumococci, and the bacterium of plague are highly invasive. Other organisms cause disease primarily through production of poisons or toxins; that is, they are **toxigenic**. The diphtheria bacillus, for example, possesses little invasive power, usually remaining strictly localized in the upper respiratory tract, but it produces the potent diphtheria toxin, which is widely distributed in the blood stream and injures tissues in remote regions of the body. Most pathogens possess both invasive power and toxigenicity to some degree. A few organisms, however, injure the host entirely through their toxins. *Clostridium botulinum*, for example, lives saprophytically in the soil and produces toxin when it grows in foodstuffs. The disease botulism results from ingestion of this toxin preformed in the food. Such organisms are not virulent in the sense of infection, since they do not invade the tissues.

The causative agents of the common infectious diseases, such as diphtheria, scarlet fever and typhoid fever, are highly virulent, true pathogens which characteristically produce disease in normal individuals. Other organisms that are less virulent behave in the manner of **opportunists** in that they produce little or no injury except when tissue damage is already present or they enter an unusual location. The latter organisms are of great medical importance as secondary invaders in wounds or following infection by more virulent pathogens. Opportunists frequently infect wounds, burns, ulcers, etc., and increase the injury to the host. Pneumonia due to secondary infection is frequent following influenza and measles.

**Variation in Virulence.** Microorganisms vary greatly in virulence. In general, freshly isolated cultures are more virulent than old laboratory strains. Naturally occurring strains of the same disease agent, however, are not equally virulent and the same strain does not maintain constant virulence. Alterations of virulence probably proceeds very slowly in nature but may be brought about more rapidly in the laboratory. Virulence may be lost by growth under unfavorable circumstances, such as cultivation on artificial media, in immune serum or



high temperatures. Organisms that produce capsules or toxins tend to lose these capacities with loss of virulence, and at the same time they may undergo associated variation in colony form. Conversely, virulence may be restored by inoculation of the organism into susceptible animals. However, if the animal is one not ordinarily infected, increased virulence for the new host may be accompanied by loss of virulence for the original host. For example, smallpox virus which has been adapted to rabbits and calves by animal passage is changed to cowpox virus, which is less virulent for man. Yellow fever virus inoculated into the brain of the mouse in many successive transfers is observed to become more pathogenic for the mouse brain and less able to produce generalized disease in monkeys and man.

Variation in virulence is of practical importance. It is one factor in the great differences in severity noted in naturally occurring disease. Reduction of virulence by artificial laboratory procedures is utilized in the production of vaccines against diphtheria, smallpox, yellow fever and a number of other diseases.

**Tissue Invasion by Microorganisms.** Microorganisms may invade the tissues of the host locally in the region of their entry or they may become more generally distributed through the tissues and the blood. In the first instance spread occurs by continuity; that is, the organisms progressively invade from the portal of entry and progressively injure the tissues with which they come into contact. Invasion by continuity occurs most readily in the loose connective tissues, such as that beneath the skin, and is opposed by the more dense, firm fasciae that separate the body into planes or layers. Infections tend to spread along these fascial layers rather than to penetrate them, so that the deeper tissues are for a time protected from the infection. Spread by continuity is typical of such infections as erysipelas and gas gangrene.

In addition to their spread by continuity, disease agents may enter the lymphatic blood and be carried in these mediums to more distant regions of the body. Organisms that invade the local lymph channels are carried to the regional lymph nodes (glands). Here the defense cells of the nodes may successfully localize or destroy the organisms and prevent further spread. Infected lymph nodes become enlarged and may abscess. Tuberculosis frequently affects the lymphatics, and bubonic plague characteristically produces an abscess of the regional lymph nodes known as a bubo.

Progressive spread through the lymph channels may result in the infection of successive groups of lymph nodes and, because the lymph eventually enters the blood, invasion of the blood. Microorganisms may also enter the blood directly and, indeed, bacteria are probably present in the blood much more frequently than is recognized by culture methods. Bacteria may be temporarily present in the blood without producing actual blood infection; this condition is known as **bacteremia**. Organisms may be carried in this way to distant regions of the body. In **pyemia**, organisms are repeatedly seeded into the blood from local abscesses and are carried to new locations in the body where they also produce abscesses. **Septicemia** indicates actual blood infection.

accompanied by multiplication of organisms in the blood. The distinction between bacteremia and septicemia is a fine one, and in many instances it is impossible to decide which condition exists.

**The Invasive Power of Bacteria.** The damage to body cells produced by bacteria is caused in large part by chemical substances which interfere with the activities of the cells, and the invasive power of bacteria results from the production by the microorganism of a variety of substances, known and unknown, that disrupt the defense mechanisms of the host. Before the nature of any of these substances was understood they were named **aggressins** to indicate the activity against the host. At present, some of these substances appear to be identical with the bacterial exotoxins, but others are probably unrelated to the poisons. It should be clearly understood that the invasiveness of any pathogenic microorganism is not solely dependent upon one property, but rather results from the interaction of several factors.

One such factor acts like an enzyme in destroying hyaluronic acid, a component of the intercellular substance uniting the body cells. The spreading factor (**hyaluronidase** or **Duran-Reynals factor**) is formed by a number of bacteria and, in addition, occurs naturally in certain animal tissues. The destruction of hyaluronic acid permits wide spread by continuity, thus increasing the size of the area of infection. The activity of hyaluronidase is nonspecific in that it increases the size of lesions produced by many bacteria and favors the spread of dyestuffs, etc.

Pathogenic bacteria produce a number of substances that act upon the blood or blood products. One of these, streptococcal **fibrinolysin** or **streptokinase** causes the clot of normal human blood plasma (the clear fluid after removal of the blood cells) to be dissolved within a few minutes, but is quite inactive against animal plasmas. The activity of streptokinase appears to be that of an enzyme-activator upon a trypsin-like, protein-digesting enzyme normally present in the blood plasma. The actual dissolution of the clot is, then, thought to be brought about by the plasma enzyme. The plasma of convalescent persons forms a clot resistant to fibrinolysis. The role of streptokinase within the body is poorly understood; however, its formation by human strains of hemolytic streptococci, its presence in body fluids and exudates during infection and the development of resistance to lysis during convalescence suggest a relationship to human disease. Substances other than fibrinolysin which slowly dissolve or prevent the formation of blood clots are produced by a number of bacteria. Since the body forms fibrin, *i.e.*, a clot, in the region of infection as a defense measure, destruction of this clot by microorganisms would seem to favor tissue invasion and spread through the lymphatic channels.

Pathogenic staphylococci produce a substance, **coagulase**, that causes coagulation or clotting of blood plasma. The role of coagulase in disease production is also uncertain, although the great majority of pathogenic staphylococci produce it. The clotting of plasma theoretically should favor localization of



organisms and thus may aid in development of the local abscesses typical of phyllococcal infections.

Both coagulase and fibrinolysin may readily be demonstrated in the laboratory. Active coagulase-producing strains of staphylococci cause clot formation in the test tube within three hours when added to small amounts of oxalated rabbit plasma and incubated at  $37^{\circ}\text{C}$ . In the usual fibrinolysin test streptococcus cultures or filtrates of cultures are added to a 10 per cent solution of oxalated normal human blood plasma, and the mixture is allowed to clot by the addition of calcium chloride. At  $37^{\circ}\text{C}$  the clot is liquefied within a few minutes in a positive test. In contrast to the rapid liquefaction of the clot of normal plasma, that of immune plasma fails to liquefy in many hours.

Many pathogenic bacteria produce **hemolysins** that destroy or lyse red blood cells and **leucocidins** that kill the white blood cells or leucocytes. Both the hemolysins and leucocidins are important bacterial antigens (see below) and at least in some instances are related to the bacterial exotoxins. Destruction of the leucocytes would appear to be advantageous to the microorganisms since these cells are important in the defense against infection. The role of the hemolysins in disease production is not well understood.

**Bacterial Hemolysins.** Bacteria may destroy red blood cells in several ways. The soluble hemolysins are found in the culture medium in the absence of the living bacterial cells and are closely related to the exotoxins. These hemolysins are demonstrated by incubating a mixture of the bacterial culture filtrate with a suspension of the washed red blood cells from a suitable animal. If hemolysin is present the hemoglobin will be liberated from the cells, giving a clear red color to the mixture. Different bacterial hemolysins are active against the red blood cells of different animals, and some bacteria produce more than one hemolysin. For example, staphylococci produce two hemolysins which are active against the red blood cells of different animals and which are antigenically distinct. The soluble hemolysins when injected into animals stimulate the formation of antibodies known as **antihemolysins**.

In addition, the colonies of certain bacteria on blood agar are surrounded by clear zones in which there is destruction of the blood. Some bacteria, such as the *alpha* hemolytic streptococci, produce complete destruction of the red blood cells with loss of color of the medium, so that the colony is surrounded by a clear zone. The *alpha* or viridans streptococci cause partial destruction of blood and are surrounded by a zone of greenish discoloration. The production of change in blood media, hemolysis, is characteristic of a number of saprophytic as well as pathogenic bacteria and is not clearly related to virulence or to the production of soluble hemolysin.

**Bacterial Capsules.** The presence of a capsule seems definitely to be associated with the pathogenicity of certain organisms. For example, encapsulated strains of pneumococci, Friedländer's bacillus, and the bacillus of anthrax are virulent, whereas the nonencapsulated strains are avirulent and are highly susceptible to destruction by the defense cells of the body. As has already been

indicated, the bacterial capsules are for the most part composed of polysaccharides, *i.e.*, complex carbohydrate materials, although the capsule of the anthrax bacillus, which is a polypeptide, protein-like substance, is a notable exception. In comparison to nonencapsulated strains of the same microorganism, encapsulated bacteria are remarkably resistant to phagocytosis by the defense cells of the host.

**Bacterial Toxins.** Microorganisms may injure the host by means of toxic substances or poisons as well as by invasion of the tissues. These toxins may either be elaborated by the organisms into the culture medium or be found within the cell substance itself. The cells of most microorganisms are more or less toxic to the animal body, and undoubtedly produce injury by their chemical effects. Only a relatively few bacteria produce soluble toxin or exotoxin.

**Exotoxins.** A number of bacteria produce toxins which are separable from the bacterial cells and which may be found in the surrounding culture medium or in the body tissues. These are known as exotoxins. Bacterial exotoxins are largely responsible for the symptoms and pathology in diphtheria, tetanus, botulism, gas gangrene and scarlet fever. The toxins, which are generally distributed throughout the body in the blood, may exert their ill effects at a distance from the site of infection and injure the body in characteristic ways. For example, the diphtheria toxin affects particularly the heart, liver and kidneys in man and produces characteristic hemorrhages in the adrenal glands of the guinea pig; and tetanus toxin injures the motor nerves. The other exotoxins produce equally specific types of injury. Present knowledge of these effects is discussed in the sections devoted to the particular disease agent.

Crude exotoxin, prepared from broth cultures by filtering off the bacterial cells, is an extremely potent poison. A fraction of a milliliter of crude diphtheria toxin, for example, is sufficient to kill a guinea pig. Such toxins may, indeed, be standardized by their lethal effect on animals. The minimum lethal dose, or MLD, of diphtheria toxin is defined as that amount of toxin which, when injected subcutaneously, is just sufficient to kill a guinea pig weighing 250 gm on the fourth day after inoculation.

The crude bacterial toxin contains the culture medium and other products of growth in addition to the toxin itself. The toxin may be greatly purified and separated from these other substances by the chemical methods used for the purification of proteins. Indeed, the exotoxins react in general like the proteins, such as egg albumen. Like the proteins they may be precipitated from solution by suitable concentrations of the salt, ammonium sulfate; they are destroyed or denatured by heat; and, with the exception of botulinum toxin, they are inactivated by the proteolytic enzymes, pepsin and trypsin. The exotoxins are also good antigens and when injected into animals stimulate the formation of a specific antitoxin which neutralizes the toxin. Recently, botulinum and tetanus toxins have been crystallized, and diphtheria toxin has been obtained in a highly pure state. Analyses of the pure substances have confirmed the complex protein nature of toxin. They have been found to be composed of amino acids and an



highly antigenic. Infinitesimal amounts of pure toxin suffice to kill experimental animals.

The discovery that toxin treated with formalin loses its toxicity is the basis of preventive immunization against such diseases as diphtheria and tetanus. A toxified toxin or **toxoid** differs from toxin in that it does not injure the animal tissues. Toxoid is protein in nature, and, of great practical importance, it retains the ability to stimulate formation of antitoxin in the animal body. Individuals immunized with toxoid are resistant to the harmful effects of toxin and hence protected against disease. Both toxin and toxoid are neutralized by or combine with antitoxin. The neutralization of toxin by antitoxin is of great value in that antitoxin may be used in the treatment of human disease. The combination of toxin with antitoxin is quantitative; that is, if one unit of antitoxin neutralizes a given amount of toxin, 10 units will be required to neutralize 10 times that quantity of the same toxin. Purified toxin also forms toxoid when treated with formalin.

**Endotoxins.** The endotoxins are so named because they are found within the bacterial cell itself, and are liberated only when the cell is destroyed. Endotoxins may be liberated naturally in old cultures which have undergone autolysis, or may be produced artificially by mechanical destruction of the cells. They may be liberated in the body when organisms are broken up or destroyed. The endotoxins are much less potent poisons than are the exotoxins, and differ from the latter in many other respects. Since they are a part of the bacterial cell, they are not found in the culture medium in large quantities. Many of them are not proteins but complex chemical compounds, the toxic portion of which has been found to be carbohydrate. Toxoid is not formed upon addition of formaldehyde. Endotoxins tend to resist destruction by heat, some surviving boiling for many minutes. They are not good antigens, and do not readily stimulate formation of antitoxin. Nonetheless, they are harmful to the body, and are an important factor in the causation of disease by bacteria.

The exotoxins and endotoxins may be compared as follows:

PROPERTY	EXOTOXIN	ENDOTOXIN
Location	Outside the bacterial cell	Within the intact cell
Toxicity	Highly poisonous	Less poisonous
Chemistry	Protein in nature	May be protein; some are complex compounds containing carbohydrates
Formalin	Toxoid formed	Toxoid not formed
Destruction	Heat; proteolytic enzymes	Resistant to heat and proteolytic enzymes
Antigenicity	Stimulate formation of antitoxin	Poor antigens

## RESISTANCE TO INFECTION— IMMUNITY

Immunity or resistance to infection has long been known to follow an attack of infectious disease and, with the development of knowledge about disease agents, it has been possible in many instances to duplicate such resistance by **vaccination** or artificial immunization. Such immunity is known as **acquired immunity**, and is present only in individuals who have been specifically immunized by disease or artificial methods. Acquired immunity is specific against the particular disease agent and in large part results from the presence of **immunoglobulins** or **antibodies** in the blood and tissues of the individual animal or human being.

Acquired immunity thus represents an unusual, enhanced ability to resist disease agents. It is less generally recognized that the normal individual also possesses a lesser, but definite, ability to resist microbial attack and that in the cases of both the normal and immune individual the resistance to disease agents is a part of a more general defense mechanism of the animal body against harmful foreign agents. Thus a variety of chemical compounds, chiefly proteins, as well as infectious agents, stimulates the formation of antibodies. In the normal individual resistance to infection is in part the result of **natural** or **inherent immunity** and is in part due to mechanical and physiological barriers against invasion by microorganisms. Similarly, foreign particles and chemical substances are prevented from entering and are removed from the body by the mechanisms which protect against invasion by microorganisms.

**Mechanical and Physiological Barriers to Infection.** All parts of the body directly or indirectly in contact with the external environment are subjected to contamination with untold numbers of microorganisms, only a few of which are able to penetrate into the deeper tissues. Invasion is opposed by the covering membranes or epithelium of the body surfaces. The epithelium of the skin covers the outer surface of the body, and that of the mucous membranes forms the lining of the respiratory, gastro-intestinal and genito-urinary tracts. These covering membranes are to a great extent mechanical barriers to infection, but in part they actively remove and aid in the destruction of microorganisms.

Secretions such as the tears, saliva and mucus, which bathe many epithelial surfaces, carry away large numbers of organisms. For example, the majority of



spired bacteria become trapped in mucus and are removed before they reach the bronchi and lungs. Infection, foreign bodies and noxious chemicals cause an increase in secretion, of which the tearing of the conjunctivae and the nasal secretion of the common cold are examples. Removal of organisms from some areas, particularly the respiratory tract, is aided by the movement of small hair-like projections of the epithelial cells known as cilia. The cilia remove many bacteria from the bronchi and lungs into the throat or pharynx from which they may be eliminated.

Certain of these body secretions are bactericidal. The acid of the gastric juice kills many bacteria but, in the absence of secretion or when the acid is neutralized by food, organisms are able to pass into the intestine where the alkaline reaction is more favorable to their growth. The normal vaginal secretion is also highly acid and is bactericidal to most bacteria. Although important, these mechanical and physiological barriers provide only partial protection against invasion by pathogenic organisms. Their proper function may be impaired by factors such as malnutrition and chilling, which reduce the general health of the host.

**Antibiotic Substances in Body Fluids.** The physiological bactericidal effect of the body fluids is not limited to the acid secretions. Several bactericidal substances have been described. One such substance, **lysozyme**, has been described from human tears and mucus, and in addition has been found in egg white. Lysozyme is bactericidal for only a few saprophytic bacteria and is inactive against pathogens and organisms of the normal flora. The activity of lysozyme seems to be due to destruction of mucoids, a component of the bacterial cell.

Two other inhibiting substances have been described in human secretions: one, known as **inhibine**, is found in human sputum and is active against a number of bacteria; the other, named **virus inactivating agent (V.I.A.)**, occurs in the nasal secretion and is able to destroy certain of the viruses.

In general, the activity of these substances is limited to only a few microorganisms and seems to be of little value in resistance to disease agents.

**General Health and Immunity.** The importance of good general health to resistance against infection is often not fully appreciated. Its role becomes apparent when infection complicates such noninfectious diseases as diabetes mellitus, the degenerative circulatory diseases and silicosis. Pneumonia is a common terminal event in the aged, debilitated patient. Tuberculosis is a particularly frequent complication of silicosis, a lung disease resulting from the inhalation of silica dust. Chilling, excessive fatigue and malnutrition also reduce the ability of the body to combat infection.

The importance of nutrition to resistance was early recognized in the association of epidemics or "plagues" to famine. More recently the reduction in resistance accompanying less severe dietary deficiency and the absence of adequate amounts of certain specific substances has been demonstrated. Deficiency of certain vitamins, notably A and C, has been found to predispose to infection

in man and animals dependent upon an external supply of these substances. The dietary proteins have been found to be of primary importance to the body defenses. The dietary proteins supply the building stones (*i.e.*, amino acids) for the body proteins and hence of the antibodies which are protein in nature. Impairment of antibody formation, of course, results in reduction in resistance to infection. The dietary proteins individually seldom contain all of the needed amino acids in sufficient quantity for good nutrition. For this reason an adequate intake is assured only when the proteins in the diet are varied.

**Species Immunity.** The resistance of some animal species to parasites that infect another species is called **species immunity**. Man is resistant to many parasites of lower animals and, conversely, animals are resistant to many human pathogens. The human malarial parasites, for example, naturally produce disease only in man; the virus of poliomyelitis may be transmitted to a few animals but is restricted in host range; typhoid fever is not reproduced in animals. The bacterial toxins, likewise, injure only certain animals. The food poisoning toxin of staphylococci produces vomiting only in man and a few animals.

The mechanisms of species immunity are for the most part poorly understood. Early in the history of bacteriology, however, Pasteur demonstrated one factor when he showed that the naturally resistant chicken could be infected with anthrax if its body temperature were lowered to that of mammals. In general, the pathogens of cold-blooded animals do not infect mammals, although a number of disease agents multiply in and are transmitted by insects and ticks. Birds, on the contrary, have been found to harbor several pathogens common to the mammals, including man. Species immunity to a given parasite usually affords a high degree of protection against that agent, but it does not imply a similar resistance to other parasites. Indeed, the animals are reservoirs of infection for man in the case of many diseases, such as bubonic plague, tularemia, psittacosis and spotted fever.

**Racial Immunity.** The resistance to infection of races or breeds of a generally susceptible species is termed **racial immunity**. Racial immunity, like species immunity, implies the inheritance of characteristics or factors that predispose to resistance. It has been possible to breed strains of laboratory animals which are unusually susceptible or resistant to certain disease agents, and in these highly inbred races there is some evidence of the inheritance of factors for resistance or susceptibility.

The effect of inheritance in naturally occurring disease is more difficult to determine. Before attributing well known differences in the numbers of cases and deaths from a particular disease directly to innate or inherited resistance, various other factors should be considered. For example, previously unexposed peoples suffer severely upon exposure to an infectious agent, a fact demonstrated by the extremely fatal epidemics of measles among certain islanders. On the contrary, long association between parasite and host populations tends to result in less severe disease. Massive exposure from infected associates, acquired



munity, and social, economic and environmental factors also play a role in the severity of infection.

Differences in the racial incidence and severity of human tuberculosis are well known. The nonwhite population particularly suffers from this infection. And some groups among the white race are more severely affected than are others. The relative roles of racial immunity and other factors are in this, as in other diseases, difficult to evaluate.

**Relationship of Age to Immunity.** Many infectious diseases tend to occur more frequently in one than in other age groups of the population. Measles, whooping cough, chickenpox, mumps, poliomyelitis, scarlet fever and diphtheria are most frequent among children between two and fifteen years of age. The incidence of tuberculosis is greatest among young adults. Mortality from pneumonia is notoriously high among the very young and the very old. Among the animals there are also many instances of a high susceptibility to infectious agents in the embryo and the newborn, the young often being susceptible to disease agents to which the adult is resistant. Indeed, the unusual susceptibility of the chick embryo to viral and rickettsial agents of disease is used extensively in the study of these agents and in the preparation of vaccines for human use.

Variability in age resistance to infection may result from many causes. The very young infant is less able to develop acquired immunity than are older children or adults. This inadequate defense mechanism may in part account for the unusual susceptibility of the newborn infant to certain infections. The infants of mothers immune to measles, diphtheria, etc., are, however, partially protected against these diseases during the first few months of life by antibodies transferred from the mother. This passive immunity is soon lost, leaving the pre-school and school child particularly susceptible at an age when there is ample opportunity to acquire infection from other children and adults. The resistance of adults to the contagious diseases of childhood is largely the result of acquired immunity from previous disease or subclinical infection. Indeed, adults who have previously escaped these diseases may develop them during exposure. There is no proof that physiological maturity alone confers immunity. The impaired circulation and general debility of the aged contribute to their lack of resistance to pneumonia and other infections.

## ANTIGENS AND ANTIBODIES

The development of resistance in the animal body following infectious disease or artificial immunization results largely from production of immune bodies (antibodies) which, by reacting or uniting with the microorganism or toxin, aid in the defense of the host. The production of antibodies is stimulated by a variety of substances collectively known as **antigens**. An understanding of the nature of antigens and of antibodies is important in understanding the immune response to infection.

**The Nature of Antigens.** An antigen (Gr., *anti*, against; *geneo*, produce) may be defined as any substance which, when introduced into the animal body, stimulates the formation of specific antibody. Antigens are usually protein in nature, are foreign to the animal body, are soluble in the body fluids and, either within the body or in the test tube, react, that is, unite, with specific antibody. Although antigens are usually proteins, only the complete proteins, such as albumen, readily stimulate antibody formation, whereas proteins which are somewhat deficient in chemical composition, such as gelatin, are relatively poor antigens. Furthermore, antibodies are readily demonstrated only against antigens which are not ordinarily present in the blood or tissues of the animal. Although the body proteins are complete proteins, and readily stimulate antibody formation when injected into another animal species, antibodies are not demonstrable against the body proteins of the same animal. Thus the rabbit may be immunized against the blood serum of the horse and human beings produce antibodies against horse serum following use of this material in serum treatment, but antibodies against rabbit serum are not demonstrated in the rabbit and antibodies against homologous body proteins are not demonstrated in man. Each pure antigen is specific and stimulates production of antibody against itself but not against other antigens. This specificity is related to chemical structure, so that chemically related antigens stimulate production of similar antibodies. Substances possessing all of the properties of antigens both stimulate the production of and react with antibody and are known as **complete antigens**.

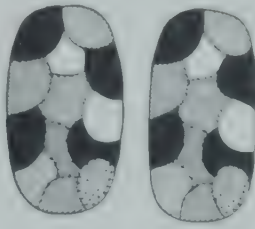
Another group of substances, known as the **haptenes**, or partial antigens, possess some but not all of the properties of antigens. The haptenes alone do not stimulate antibody formation, but in combination with a complete antigen alter the specificity of the resulting antibody, and react with it in the test tube. The haptenes in general are simpler substances than are the complete antigens and are not protein. The best known natural haptenes are perhaps the carbohydrates of the pneumococcus capsules. These capsular substances, also known as Soluble Specific Substances (SSS), are different for each pneumococcus type and their injection together with the pneumococcus cells causes formation of type-specific antibodies against the capsular hapten. Antibodies produced against rough, nonencapsulated pneumococci are not type specific. Bacteria of various species contain haptenes as a part of the cell or the capsule, which lend specificity to the antibodies against the organism.

**Bacteria as Antigens.** The bacteria are complex structures containing a number of separate substances all of which may act as antigens. The bacterial antigens are located within the cell itself and, in those bacteria which possess them, in the flagella and capsules. For purposes of immunology the bacterial cell is often looked upon as a mosaic or pattern of many different antigens, such as is illustrated in Fig. 135. The antigens are part of the protoplasm of the cell. In view of their complex structure it is not surprising that different bacteria sometimes possess similar or identical antigens. This sharing of antigens is from

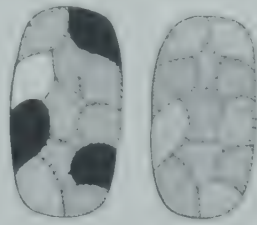




THE BACTERIAL CELL HAS MANY ANTIGENS IN A MOSAIC PATTERN: BACTERIA WHICH HAVE FLAGELLA OR CAPSULES ALSO HAVE ANTIGENS IN THEIR STRUCTURES



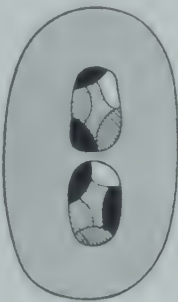
THE ANTIGENS OF IDENTICAL BACTERIA ARE ALL ALIKE



RELATED BACTERIA HAVE SIMILAR OR SOME IDENTICAL ANTIGENS - BUT OTHER ANTIGENS ARE DIFFERENT



BACTERIA (e.g. *PNEUMOCOCCI*) MAY HAVE THE SAME CELLULAR ANTIGENS BUT DIFFERENT CAPSULAR OR FLAGELLAR ANTIGENS



DIFFERENT BACTERIA MAY POSSESS THE SAME OR RELATED CAPSULAR AND FLAGELLAR ANTIGENS



Fig. 135. Antigenic structure of the bacterial cell. The differently shaded areas indicate chemically different antigens.

quently encountered among closely related bacteria, but some otherwise unrelated organisms have similar antigens. Although bacteria may contain some related antigens, only identical organisms contain *all* of the same antigens.

**The Nature of Antibodies.** Injection of an antigen into the animal stimulates it to produce antibody, which may be demonstrated in both the tissue and blood of the immune animal within 7 to 14 days after inoculation of antigen. Usually antibody is obtained in the blood serum. Such serum, known as **immune serum** or **antiserum**, reacts with the specific antigen. Serum antibodies are proteins closely related to the normal serum globulins and, indeed, the serum globulin is increased in immune animals. Antibody globulin may be recognized by its reaction with antigen, a property not possessed by normal globulin. Physico-chemical study of serum proteins has yielded additional information about the nature of antibodies. Thus, in the separation of serum globulin into euglobulin and pseudoglobulin fractions by chemical methods, antibody protein may be precipitated with either fraction. Precipitation with normal serum globulins provides a useful method for concentrating and partially purifying antibody. Recently methods have been developed for studying proteins by an electro-chemical method known as electrophoresis. Examination of the blood serum of immune animals and man by this method has revealed that antibody is generally associated particularly with one fraction, the gamma globulin. Gamma globulin is increased in the immune individual, whereas the other components, the alpha and beta globulins, are not increased.

Since antibodies are known principally by the specific reactions with antigens which stimulated their production, it is customary to name the antibodies according to these reactions. As will become evident, many of the reactions result from a single antibody in combination with its antigen, and not from different antibodies against the same antigen. According to this nomenclature the most commonly recognized antibodies are:

The **precipitins**: Antibodies which form a precipitate with soluble antigen.

The **agglutinins**: Antibodies formed against cells, such as bacteria, which when mixed with these cells cause them to clump together or agglutinate.

The **antitoxins**: Antibodies formed against poisonous toxins, such as the bacterial exotoxins, which specifically neutralize the effects of the toxin.

The **lysins**: Antibodies which cause the death and dissolution of bacteria and other cells.

The **bactericidins**: Antibodies which cause the death of bacteria, but not their dissolution. The bactericidins are probably identical with the bacteriolysins.

The **opsonins**: Antibodies which sensitize the cells of microorganisms so that they are readily ingested or engulfed by the phagocytic cells of the body.

The **protective antibodies**: Antibodies which, when combined with pathogenic organisms, render them noninfectious. Protective antibodies are studied particularly against the cytotropic viruses.

**The Reactions of Antigen and Antibody.** The reaction of an antigen with its antibody is a chemical union between the two substances. Ehrlich looked upon this union as a firm chemical union, such as that between a strong acid



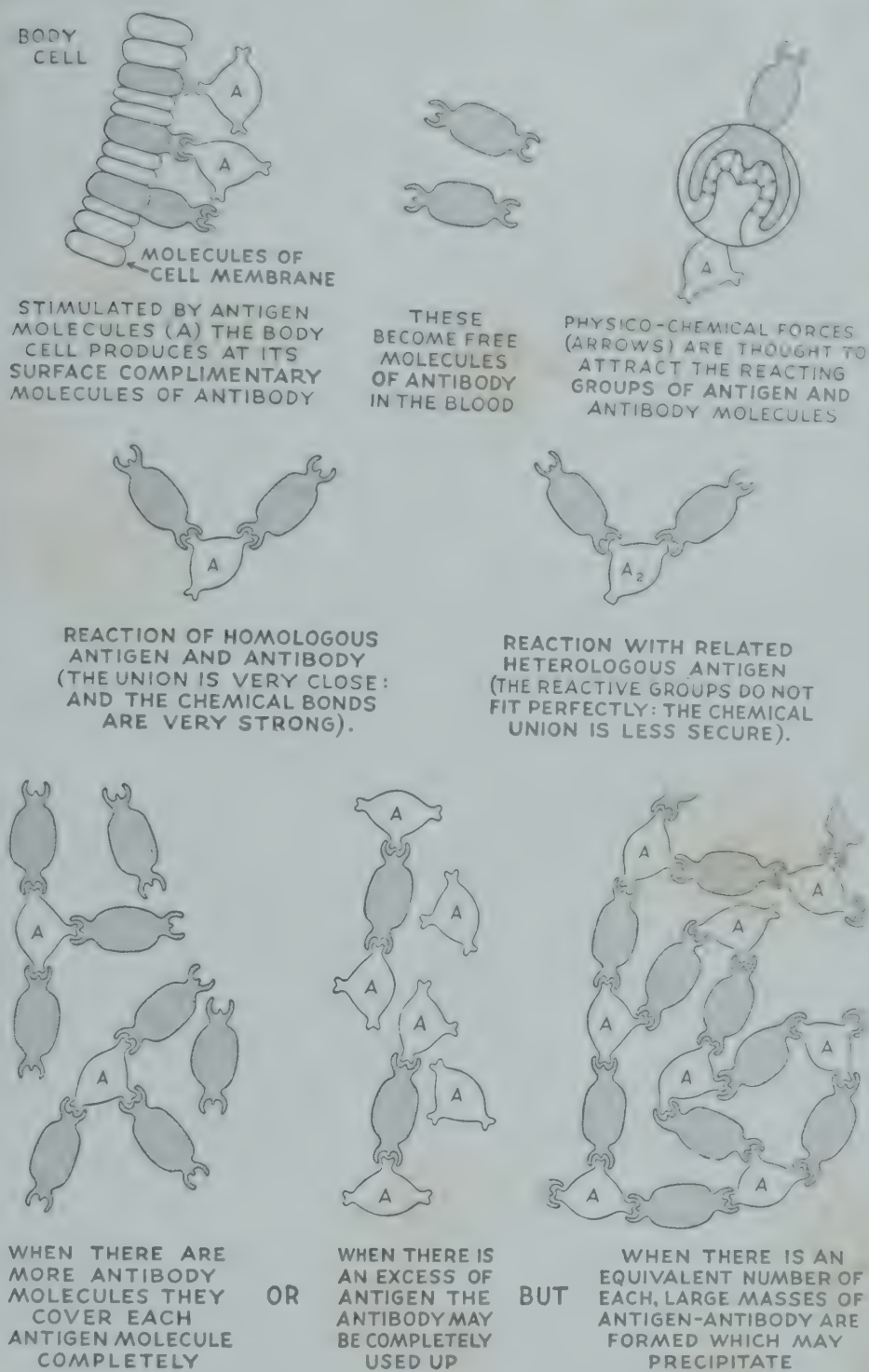


Fig. 136. Diagram of the concept of formation of antibody by the body cell (Felix) and the reaction of antigen and antibody as in the precipitin reaction.

and a strong base. The molecules of antigen may be thought of as fitting chemical structure of the antibody somewhat as a key fits into a lock. Ehrlich described the formation of antibody as the production of little side-chains on a surface of the cells which, becoming broken off, circulated in the blood as antibody molecules. A diagram of Ehrlich's side-chain theory is shown in Fig. 135. Other immunologists have likened the antigen-antibody reaction to that of a weak acid and weak base or to an adsorption of the antigen molecules by the antibody.

According to the modern view, the antigen-antibody reaction is a highly specific chemical union in which the molecules are held together by strong attractive forces. The antibody molecules (the side-chains of Ehrlich) are large complex proteins with specific groups on their surfaces (the locks) which combine chemically with complementary groups (the keys) of the antigen. It is thought that antigens may have many such groups and that the antibody molecule has at least two combining groups. It is thus possible by the reaction of many antibody molecules to build up a large mass or lattice work of interlocking antigen-antibody units (Fig. 136). Under suitable conditions this mass may become sufficiently large to form a visible precipitate or, in the case of bacteria, the cells may become clumped together. The combination of antigen and antibody may, however, give rise to a number of different effects or reactions depending upon the nature of the antigen and the conditions of the reaction.

**The Precipitin Reaction.** The precipitation of an antigen with its specific antibody, known as the **precipitin reaction**, is one of the most valuable and widely used immunological reactions. The test is performed either by the ring test in which the antigen solution and serum containing the **precipitin** are layered in the test tube, or by mixing the antigen and serum. In either test a positive reaction is indicated by the formation of clouding or precipitate of the antigen and antibody. The precipitation is quantitative, and by dilution methods a titration or endpoint, may be obtained which indicates the strength or potency of the serum. Precipitins may be demonstrated against extracts of bacterial cells, bacterial haptenes, toxins, the blood proteins and numerous other natural and artificial antigens.

**The Agglutination Reaction.** The **agglutination reaction** has the same general characteristics as the precipitation reaction, except that it involves the clumping together of cells rather than the precipitation of molecules from solution. The test is widely used in the identification of bacteria and is of value in the diagnosis of such diseases as typhoid fever (the Widal reaction), tularemia and brucellosis. It is also used in the typing of blood.

The agglutination test may be performed in two ways: the macroscopic tube test and the microscopic test. In the former a constant amount, usually 0.5 ml., of a dilute standardized suspension of bacteria or other cells is added to increasing dilutions of the agglutinating immune serum. The tubes are then incubated in a water bath and observed for the formation of clumps of the cells which settle to the bottom of the tube. The microscopic test is performed



ing a loopful of concentrated serum (dilution 1:10 to 1:40) and a similar loopful of bacterial culture. The preparation is then viewed under the microscope showing clumping of the cells. A macroscopic agglutination test is illustrated in Fig. 137. The appearance of the microscopic test is shown in Fig. 138.



Fig. 137. The macroscopic agglutination test. Tubes numbered 6, 8 and 10 show coarsely granular agglutination, whereas Tube 11 contains too high a dilution of serum to cause agglutination and Tube 14, the antigen control, contains no serum. Tube 15 contains only saline and is included to show the density of the bacterial suspension in the other tubes. The test shows the coarse flagellar agglutination of *Salmonella typhimurium* and specific antiserum.

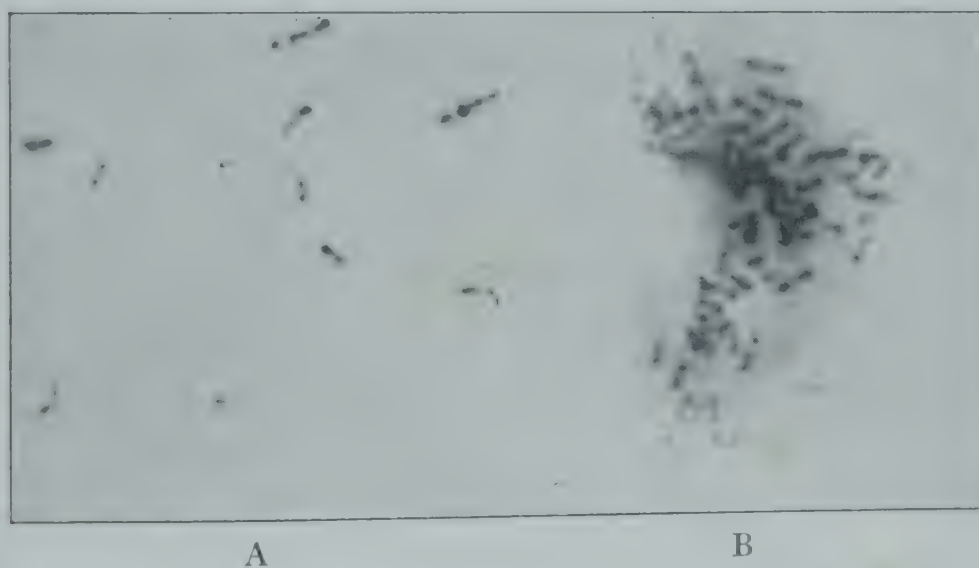


Fig. 138. The microscopic agglutination test. In (A) pneumococci are shown evenly distributed over the microscopic field, whereas in (B) they are shown agglutinated into a large cluster following addition of antiserum. The slides were fixed and stained for photographic purposes and the mass of precipitate surrounding the agglutinated bacteria retained some of the dye.

*The Lytic Reaction.* The first observation of the disintegration of cells was made by Pfeiffer, who noted that cholera vibrios inoculated in peritoneal cavity of immune guinea pigs rapidly died and completely appeared. Lysis in the presence of fresh blood serum or other body fluids of immune animal was soon found to occur outside the body and was demonstrated against red blood cells as well as microorganisms. The immune bodies responsible for the disintegration of bacterial cells are generally referred to as **bacteriolytic** and those active against the red blood cells are named **hemolysins**.

The lytic reaction, unlike precipitation or agglutination, requires the presence not only of the antigen and antibody but also of a third substance which is present in the blood. This substance, known as **complement**, is not an antibody. Complement is present in normal as well as in immune blood, is not increased by immunization and is destroyed by heating at  $55^{\circ}$ – $56^{\circ}$  C for 30 minutes. Complement, like the antibodies, is protein. It is found in the serum of many animals, but in practice it generally is obtained from the guinea pig.

The mechanism of the lytic reaction is not clearly understood, but the evidence suggests that the cells are altered or sensitized by the antibody in a way that they become susceptible to the destructive action of complement. Neither complement nor lysin alone will produce the reaction.

The lytic reaction may be diagrammed as follows:

*Antigen (cells) + antibody (lysin, i.e., heat-inactivated serum) produces no lysis.*  
*Antigen (cells) + complement (normal serum) produces no lysis.*  
*Antigen + antibody + complement produces lysis.*

The immune lysis of susceptible bacteria and that of red blood cells appear to be quite similar reactions, differing of course in the specific cells and antibodies involved in the reactions.

In the positive hemolysin test a clear red color develops as a result of the liberation of hemoglobin from the disintegrated red blood cells. In the negative test the cells remain intact and settle to the bottom of the tube, leaving the liquid colorless. Bacteriolysis, on the other hand, is usually observed with the aid of the microscope. Grossly, a culture undergoing lysis is seen to lose turbidity as the bacteria are destroyed.

*The Bactericidal Reaction.* Bacteria may be killed by the combined action of immune serum and complement without undergoing lysis or any visible disruption or integration. Indeed, the killing effect of immune serum on bacteria differs from bacteriolysis only in the absence of visible disruption of the cells. Complement is required for both reactions, and the tests are similarly carried out. These reactions are, then, closely related phenomena.

*The Complement-Fixation Reaction.* Complement may be bound or fixed in many antigen-antibody reactions in addition to that of the lysis of cells or the bactericidal reaction. In some of these combinations no visible change is observed, so that detection of the reaction may be difficult. However, complement which has been bound in one reaction cannot react in a second test. It is, the



possible to demonstrate the fixation of complement by adding a second antigen, such as red blood cells and hemolysin, which does give a visible reaction. In the complement-fixation test, complement is allowed to react with one antigen-antibody system, and then an indicator system consisting of red blood cells and their hemolysin is added to the tubes. If hemolysis of the red blood cells occurs, the complement has not been fixed by the first antigen-antibody reaction and the test is negative. In the positive test, complement is fixed in the first reaction and hence is not available to produce hemolysis in the red blood

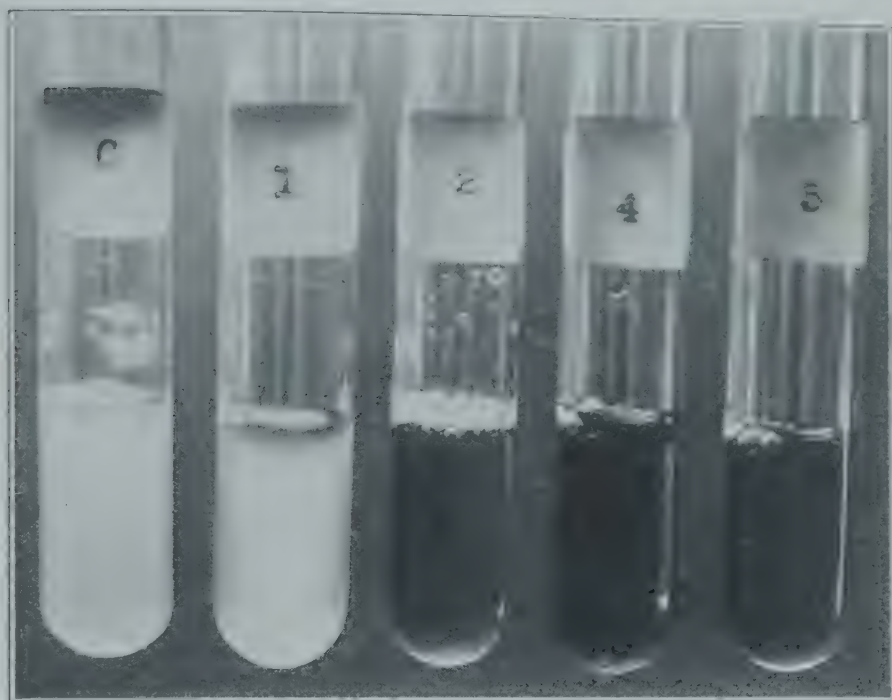


Fig. 130. The hemolysin test. The control Tube C and Tube 1 contain intact red blood cells and appear turbid. Tubes 2, 4 and 5 show marked or complete hemolytic destruction of the cells and appear clear. All tubes are of course red in color.

system. The most widely known complement-fixation reaction is the Wassermann test for syphilis (Fig. 139).

**The Protective Reaction.** The ability of antibodies to protect the animal against pathogenic organisms is determined by the protection test. The test is performed by injecting susceptible animals with a mixture of immune serum and the infectious agent. Protection against disease is specific and occurs only in those animals given sufficient antibody to **neutralize** or **inactivate** the disease agent. The protection test is an important method of determining the ability of bacterial serums, such as *antimeningococcus* or *antipneumococcus* serum, to protect against disease. It is also useful in the detection of antibodies against filtrable viruses and in the identification of these agents. Protection is not obtained if normal serum or immune serum against other organisms is given.

The protective action of immune serum undoubtedly is dependent upon the effects of antibodies upon microorganisms. Thus the effect of antiserum on

the killing, disintegration and phagocytosis of microorganisms contribute its ability to protect against infection.

**The Toxin-Antitoxin Reaction.** Antitoxin (Gr., *anti*, against; *to*, poison) was demonstrated early in the history of immunology by von Behring and Kitasato when in 1890 they described the remarkable protective action of immune serum against the toxins of diphtheria and tetanus. Like other antibodies, antitoxin is found in the blood of immune animals and reacts specifically with its antigen, in this case toxin. Antitoxin owes its protective effect to the direct neutralization of toxin. Neutralization may occur either in the body fluids or in the test tube but not after toxin has become attached to the body cells. The latter fact explains to a large extent the importance of the early use of antitoxin in the treatment of disease. The neutralization proceeds quantitatively, so that 10 times the amount of a toxin which will combine with one unit of antitoxin will combine with 10 units of antitoxin. In the test tube, toxin and homologous antitoxin, mixed in proper proportions, form a precipitate or flocculation, a reaction of considerable value in standardization.

**THE STANDARDIZATION OF ANTITOXIN.** The use of antitoxin in the treatment of disease makes its standardization particularly important. The basis of standardization is the protective action of antitoxin against the ill effects of toxin on susceptible animals.

Since both toxins and antitoxins possess variable potency, the standardization of one is dependent upon that of the other. In diphtheria, the first standard unit determined was the Minimum Lethal Dose or MLD of toxin, which has been previously defined (Chap. 22). The early unit of diphtheria antitoxin was defined by Ehrlich in terms of the MLD of toxin as that amount of antitoxin which would neutralize 100 MLD of toxin. It was soon found, however, that antitoxins standardized by this method against different toxins did not have the same potency owing to differences in the toxins. Toxins naturally contain variable amounts of toxoid, which although not toxic to the animal is able to neutralize antitoxin. Furthermore, toxins are altered by storage. The antitoxins, on the other hand, remain potent for long periods of time, and may be compared one with another against a single toxin. Therefore, one particular antitoxin was arbitrarily chosen as an international standard antitoxin.

One unit of diphtheria antitoxin is then correctly defined as that amount of antitoxin which has the same potency or combining power for toxin as 1 unit of standard antitoxin.

In the United States, unknown antitoxins are compared with standard antitoxin supplied by the National Institute of Health. The international unit was established under supervision of the Biological Standards Commission of the League of Nations, and is based upon antitoxin standardized and preserved by Ehrlich.

The actual procedure of standardization is briefly as follows. A diphtheria toxin is prepared and the amount of this toxin which when mixed with 1 unit of standard antitoxin is just sufficient to kill a 250 gm. guinea pig in 4 days is determined.



ed. This amount of toxin is known as the  $L_+$  dose of toxin. Second, the amount of unknown antitoxin which has protective power equal to the standard toxin is determined using this  $L_+$  dose of the same toxin. Since this amount of unknown antitoxin has the potency of 1 unit of standard antitoxin, it also contains 1 antitoxic unit.

Antitoxins may be compared by the flocculation (*i.e.*, precipitation) test and the skin reaction in guinea pigs. Antitoxin prevents the development of an area of redness in the skin of these animals following the injection of toxin.

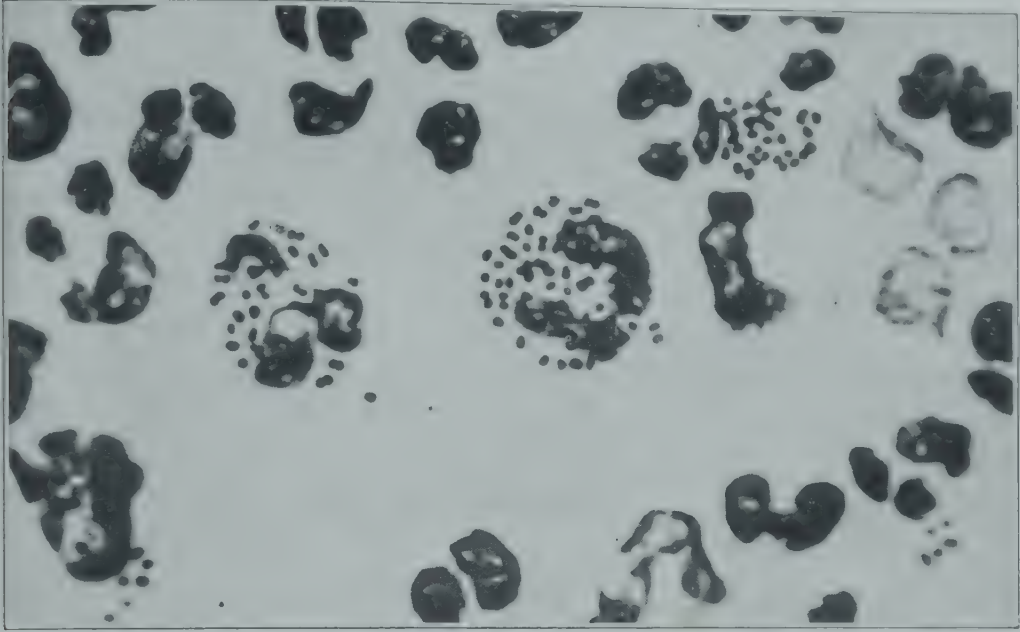


Fig. 140. The phagocytosis of bacteria. Photomicrograph of gonorrheal pus showing many gonococci within the leucocytes (Kral).

The units of antitoxins other than diphtheria antitoxin are differently defined, although international units have been established for some antitoxins, they are not available in all instances. Standardization in all cases depends upon the ability of the antitoxin to neutralize its homologous toxin.

**The Opsonic Reaction.** Bacteria, other infectious agents and nonliving particulate matter are removed from the body largely by the activity of defense cells or phagocytes (Gr., *phago*, eat; *kylos*, cell), which ingest and destroy these agents. Although phagocytic activity against bacteria is present to some extent in the normal animal, it is greatly increased by immunization. Furthermore, phagocytosis may be demonstrated *in vitro* by using suitable mixtures of the bacteria and leucocytes (one of the phagocytic cells), bacteria and blood serum. Phagocytosis is illustrated in Fig. 140. The remarkable increase in phagocytosis in the presence of immune serum is attributed to the presence of antibodies, known as **opsonins** or bacteriotropins.

The opsonins (Gr., *opson*, sauce) act upon the cells of the microorganism in such a way that they are prepared or made more susceptible to ingestion by the phagocytes. Opsonins are present to some extent in normal serum, but, as indi-

cated, they are increased by immunization. Opsonins are specific. For example, those produced against pneumococci do not increase the phagocytosis of other bacteria. Opsonic activity, like bacteriolysis, requires a heat-labile complementary substance present in the fresh serum of the normal or the immune animal. After ingestion by phagocytes, bacteria are generally killed and destroyed by the cells. Some organisms, however, are resistant to the destructive action and may survive for some time. The destruction and removal of infectious agents by phagocytic cells is an essential part of the defense mechanism and will be discussed subsequently in greater detail.

**THE OPSONIC INDEX.** The measurement of the opsonic power of serum may be carried out by comparing the amount of ingestion of bacteria by leucocytes in immune serum with that in normal serum. The leucocytes, bacteria and immune serum are mixed in small tubes and incubated for 30 minutes. The number of bacteria engulfed by 100 leucocytes in each mixture is counted on stained smears under the microscope. The ratio of the number of bacteria per leucocyte in immune serum to that in the normal serum is called the **opsonic index**. Practically, the procedure is difficult and is of limited use. A modified opsonic test is of value in the diagnosis of infection with *Brucella* organisms.

**The Unity of Antibodies.** Originally the various reactions between antigen and immune serum were thought to be due to separate and distinct substances. Thus precipitins, agglutinins, lysins, complement-fixing antibodies, etc., were viewed as separate antibodies. As evidence accumulated, however, it became more and more evident that a single antibody is probably formed against one antigen. The different antigen-antibody reactions are determined by the conditions of the test and the nature of the antigen, but they result from the union of a single antigen and one antibody. This view, known as the unitarian hypothesis, is today generally accepted as best explaining the observed facts. Microorganisms which contain many antigens, give rise to many independent antibodies, each specific for one antigen.

**Source of Antibodies.** Antibodies are produced by the animal body. The source within the body is, however, not definitely known. They undoubtedly are formed by the body cells, most likely by the defense cells known as the lymphoid macrophage system. These cells include the lymphocytes of the blood and the large phagocytic cells of the tissues. The latter are particularly numerous in the liver, spleen and bone marrow, and are widely distributed in many parts of the body. If the tissue phagocytes or macrophages are functionally blockaded, as by the injection of India ink, or if the spleen is removed, the formation of antibody is temporarily impaired. Recently, antibody has been found in the lymphocytes and the role of these cells in formation or storage of antibody has been much discussed.

**The Cells in Immunity.** In those diseases caused by toxins, such as diphtheria and tetanus, immunity depends directly upon the neutralization of toxins by antitoxin, that is, upon the direct effect of antibody. The bacteriolysins and bactericidins also aid directly in defense by killing and destroying the organism.



many infections, however, resistance is primarily cellular. The importance of cells in immunity was first appreciated by Metchnikoff, who established the theory of phagocytosis. Metchnikoff observed that certain special defense cells or phagocytes were able to ingest microorganisms and other harmful agents and to destroy or remove them from the body. Phagocytosis occurs to some extent in the normal animal, but it is greatly increased in the immune animal by the forming antibodies.

The phagocytes are widely distributed throughout the body in the circulating blood and lymph and in the tissues. In general, these cells are of two types: the microphages (the little phagocytes) and the macrophages (the large phagocytes). The microphages are the polymorphonuclear leucocytes. The leucocytes are formed primarily in the bone marrow, are normally present in the blood, and can thus be rapidly mobilized in the event of infection. The leucocytes are particularly active in bacterial infections, and they ingest and destroy many organisms. They are of little importance in diseases caused by fungi or animal parasites. The macrophages are large scavenger cells, whose function is primarily phagocytosis and the destruction of many kinds of foreign particles. These cells are strategically located in the spleen, liver, bone marrow, about the small blood vessels and in the connective tissue generally. Macrophages may be formed from undifferentiated connective tissue cells or from lymphoid cells, and they may themselves multiply by cell division. The macrophages play a major role in defense against disease agents. Their function is particularly evident in chronic infections, such as tuberculosis, and in the protozoan infections.

In addition to the phagocytes, cells known as fibroblasts are important to defense. The fibroblasts are connective tissue cells whose function is one of repair. After injury or infection they are concerned with healing, scar formation and the walling-off of harmful agents. They are not phagocytic.

**Inflammation.** The reaction of the body to local injury, whether mechanical, chemical or infectious, is termed inflammation. Inflammation represents an attempt by the body cells to wall-off and destroy the injurious agent and to repair the damage done by the agent. When this defense is successful, the injury is relatively slight; the injurious agent is kept localized and may be quickly destroyed. When unsuccessful, spread into the surrounding tissues or throughout the body may occur with production of more severe injury.

Inflammation begins with the formation of an **exudate** that is the accumulation of fluid and leucocytes from the blood about the site of injury. If the injury is sterile, few leucocytes are present, but in bacterial infection these cells migrate from the blood in large numbers. Within a short time the macrophages are mobilized and continue the attack begun by the leucocytes. The macrophages remove the debris of injured cells and tissues as well as the microorganisms. Tissue repair or healing begins early in the process, but becomes predominant as the injurious agent is destroyed and the debris is removed. When a scar is formed, healing is complete.

**Types of Inflammation.** **Acute** inflammation may be recognized generally by the redness, swelling, warmth and tenderness of the affected area. Perhaps the most common variety of acute inflammation is the boil or abscess. In this the infection has resulted in pus formation or suppuration. **Pus** is made up of fluid containing dead cells, fibrin from the exudate, leucocytes and the causative organism. Suppuration is commonly the result of infection with pyogenic bacteria such as the *Staphylococcus*. In diphtheria and certain other diseases the inflammation is **membranous** and is characterized by the presence of a toughly adherent false membrane of fibrin, leucocytes and bacteria. The fully developed lesions of anthrax typically contain blood and are referred to as **hemorrhagic** inflammation. At times, as in chickenpox and smallpox, blisters or vesicles are formed which contain largely fluid exudate. Vesicles also occur in burns and certain poisonings such as ivy poisoning. Exudates composed largely of fluid are described as **serous**.

**Chronic inflammation** results when infections continue for many days or weeks. The macrophages and fibroblasts are the principal cells in chronic inflammation. Pus may be formed, but the process is chiefly reparative with new tissue formation and scarring. The tubercle is a special variety of chronic inflammation. Fungus disease, leprosy, syphilis and many infections caused by animal parasites are also chronic.

**The General Cellular Response to Infection.** Widespread infections or invasion of the blood stream stimulate a general cellular response. In generalized bacterial infections there is ordinarily an increase in the number of the blood leucocytes, a condition known as **leucocytosis**. The degree of leucocytosis varies with the severity of the infection. In a disease such as pneumonia the number of the white blood cells may be increased from the normal 5,000 to 10,000 cells to as many as 20,000 to 30,000 cells in each cubic millimeter of blood. The leucocytosis is commonly accompanied by increased activity of the macrophages and lymphoid cells of the spleen, liver, bone marrow and lymph nodes. Thus the regional lymph nodes typically enlarge during infection, and the spleen may be moderately increased in size, as in typhoid fever, or enormously enlarged as in malaria.

Both local inflammation and the general cellular activity against infection aid the body in combating disease agents. In general a satisfactory cellular reaction suggests a favorable outcome of the infection, whereas its absence in infectious diseases which usually stimulate an increase of phagocytic activity is of great significance.

## NATURAL AND ACQUIRED IMMUNITY

The existence of two types of immunity, **natural** or innate and **acquired** or that incited against a specific agent, has been mentioned as aiding in resistance to infection. These two types of immunity may now be more completely discussed.



**Natural Immunity.** Numerous mechanisms, among them mechanical and physiological barriers to infection, species and racial immunity and naturally occurring antibodies, aid the normal host in resistance to microorganisms. Of these mechanisms the mechanical and physiological barriers are nonspecific. The activity of certain secretions, unfavorable body temperatures, physiological anti-toxic substances, ciliary action, etc., are in general active against a variety of microorganisms. Furthermore, the defense cells of the normal animal ingest and destroy large numbers of microorganisms and remove other particulate material. On the other hand, normal blood is often bactericidal and may contain specific antibodies, such as agglutinins, in low titer.

A few natural antibodies are inherited, as for example those responsible for human blood groups. The origin of other antibodies is less well understood, in many instances they are undoubtedly due to subclinical infections. In others the presence of identical or similar antigens in different organisms has been responsible. Antibodies present in the normal animal are, then, essentially similar to those of specific acquired immunity.

**Acquired Immunity.** Increased resistance may be acquired **actively** as the result of infection or artificial immunization, or **passively** as "ready-made" antibodies. In either case the immunity is specific and is of a higher grade than is natural immunity.

**Active Immunity.** Active immunity is produced by the host in response to a particular antigen, bacterial or otherwise, which has gained entrance into the body either naturally or by artificial inoculation. Following an attack of an infectious disease the individual is commonly resistant to another attack of the same disease for a considerable period of time, frequently years. This increased resistance is associated with the presence of specific antibodies and an increased activity of the defense cells. Active immunity may thus result from infection—natural or unrecognized—with a given disease agent and it may also be produced by artificial immunization.

**Artificial Active Immunization.** A great advance in preventive medicine was made when Edward Jenner noted (publication in 1798) that inoculation with the virus of the mild disease cowpox or vaccinia (*L. vacca*, cow) conferred immunity against the dread disease smallpox. Indeed, vaccination in its original strict sense may be defined as the practice of producing vaccinia by inoculation with vaccine virus in order to protect against smallpox. The principle of vaccination or artificial active immunization by the injection of antigen is today well established and is widely used against many disease agents. Suitable preparations of microorganisms or their products are available for immunization against smallpox, diphtheria, tetanus, typhoid and paratyphoid fevers, typhus and many other diseases. The immunity conferred by these agents is active immunity and appears after the lapse of a short time interval following the injection of the vaccine. Active artificial immunization for this reason is generally prophylactic or preventive measure rather than a therapeutic or curative one.

It is of little and in most instances of no value after the onset of an attack of disease.

Active immunity may be produced by inoculation of several types of vaccines or bacterial products:

(1) **Suspensions of microorganisms killed by heat or antiseptics.** Vaccines of this type are commonly prepared by suspending the microorganisms in physiological saline, after which they are killed either by heating at  $55^{\circ}$ – $60^{\circ}$  for 60 minutes or by the addition of phenol (carbolic acid) or formaldehyde. These vaccines are then standardized for uniformity and potency and are tested for sterility before being released for use. Suspensions of killed microorganisms are widely used for the immunization of man and animals. Typhoid vaccine is of this type.

(2) **Microorganisms which are attenuated or reduced in virulence.** Living organisms that have lost their natural virulence through long cultivation under unfavorable circumstances or their adaptation to a new animal host may be used for artificial immunization. Vaccination against smallpox, for example, takes advantage of the protection provided by cowpox or vaccinia against the more severe smallpox. Smallpox virus by infection of rabbits and cattle loses its virulence for man. (See Chap. 41 for the preparation of vaccine virus.) The viruses of rabies and yellow fever likewise become reduced in virulence for man by animal passage, a fact of great importance in the artificial immunization of man. Attenuated bacterial cultures are not widely used for active immunization. However, the attenuated culture of bovine tubercle bacillus known as B.C. (Bacille Calmette-Guérin) has been used by some in an attempt to protect against tuberculosis.

(3) **The products of bacterial growth.** After discovery of diphtheria toxin and antitoxin a neutral mixture of toxin and antitoxin was widely used for active immunization. Later, toxin detoxified by formaldehyde, *i.e.*, toxoid, was found to be nontoxic and to stimulate the production of antitoxin. This substance is now widely used in the artificial immunization against diphtheria and tetanus either as the soluble or the insoluble alum-precipitated product. Alum-precipitated toxoid because of its insolubility is slowly absorbed and provides a more continuous stimulus. A high degree of protection follows toxoid immunization.

In addition to the soluble toxins the products of autolyzed or disintegrated bacterial cells may at times be used for immunization.

(4) **Mixed or polyvalent vaccines:** If several antigens are inoculated at the same time, each will stimulate the production of antibody. Advantage is taken of this fact in the use of vaccines containing more than one bacterium, as for example in the case of the commonly used triple vaccine composed of typhoid, paratyphoid A and paratyphoid B bacteria (TAB vaccine). Immunization with TAB vaccine stimulates antibody formation against all three organisms. Mixed combined toxoid may also be used for artificial immunization.

(5) **Autogenous vaccines** are vaccines prepared from microorganisms isolated from an individual patient for use in that patient.



Bacteria for vaccines may be readily obtained in pure culture and the bacterial toxins may be produced relatively easily in large quantities. The filtrable viruses and rickettsiae, on the other hand, do not grow on nonliving media and with few exceptions difficult to obtain in sufficient quantity and in a suitable form for artificial immunization. Viral and rickettsial vaccines are, therefore, usually made of suspensions of infected animal tissues which are formalinized or otherwise made safe for use. In recent years it has been possible to grow a number of these agents either in flask cultures containing living cells or in the developing chick embryo and to prepare vaccines from suspensions of the virus obtained from such sources. Yellow fever vaccine, for example, may now be prepared from the infected chick embryo, as may vaccines of the rickettsiae.

TABLE 10. DIFFERENCES BETWEEN ACTIVE AND PASSIVE IMMUNITY

	ACTIVE IMMUNITY	PASSIVE IMMUNITY
Participation of the body	Active participation in antibody formation	Passive recipient of antibodies
Material introduced into body	Antigens	Antibodies
Method of acquiring immunity	Naturally by an attack of disease or subclinical infection  Artificially by inoculation with vaccines, toxoids, etc.	Naturally by transfer of mother's antibodies across placenta and through the colostrum to nursing infant  Artificially by injection of antiserum (antitoxin; antibacterial serum)
Duration of immunity	Months and years	A few weeks
Value	Chiefly preventive or prophylactic	Chiefly therapeutic or curative

Artificial active immunization or vaccination is not equally successful in all cases. Immunization with toxoid against diphtheria and vaccination against smallpox and typhoid fever confer a high degree of protection against disease. Certain microorganisms are, however, relatively poor antigens, so that vaccination provides little protection against infection with them.

**Passive Immunity.** The first demonstration of passive immunity was made in 1890 when von Behring demonstrated that the blood of convalescent animals could protect susceptible animals against diphtheria and in the sick animal would prevent death. In passive immunity antibodies produced in one animal are transferred in the serum to another individual who receives benefit from them. The

recipient animal or man does not participate in the production of the antibody. Passive immunity may be either natural or artificial. **Natural passive immunity** occurs only in the newborn animal as the result of the transfer of antibodies from the mother to the offspring either in the placental circulation during pregnancy, such as occurs in human beings, or in the colostrum to the newborn, such as occurs in cattle. In either case the newborn infant is provided with passive protection against disease agents for a short time after birth. **Artificial passive immunity** is provided by the injection of immune serums, such as antibacterial serums or antitoxins. Active and passive immunity thus differ essentially in the degree of participation of the individual in the production of immunity. They differ also in the duration of protection and in their practical applications. Passive immunity is of short duration, provides protection for only a few weeks and is of greatest value in the treatment of disease, whereas active immunity is of long duration, may provide protection for many months or years and is used in the protection of the individual against future infection. The essential characteristics and essential differences of active and passive immunity are outlined in Table 10.



# 24

## HYPERSENSITIVITY

Immunization usually provides increased protection against disease agents. It is, however, not always the case. It was early recognized that man and animals sometimes develop increased sensitivity to a disease agent or to a pure chemical substance and that this abnormal reaction generally results in ill effects on a second exposure to the same material. This condition, known as **hypersensitivity**, is most marked in the case of **anaphylaxis**, and is commonly manifest in the less severe **allergies**, such as hay fever, allergic asthma and rum sickness in man. As will become clear, the phenomena of hypersensitivity and immune reactions similar in kind to those which ordinarily protect against disease.

**Anaphylaxis.** Early in studies of immunology it was noted that animals which showed no reaction to a first injection of a serum became acutely ill and frequently died following subsequent, widely spaced injections of the same material. This condition is named **anaphylactic shock**. Anaphylaxis (from the Greek, meaning "against protection") occurs in a number of animals, including the rabbit and guinea pig, but is rarely observed in man. The cardinal features of anaphylaxis are as follows. It is a specific immune response; the animal rendered hypersensitive by an injection of an antigen is given another injection of the same antigen after an interval of 12 or more days; the second injection is followed within a few minutes by the development of shock. The initial or sensitizing dose of antigen may be injected by any route, although small doses given intravenously are most satisfactory. Indeed, the guinea pig may be sensitized by as little as one-millionth of a gram of pure protein. Actually, sensitivity may follow one or many injections provided a suitable time elapses between the sensitizing injections and the "shock" dose; hypersensitivity may be avoided by giving injections of antigen at short intervals. The use of large doses of the material at proper intervals results in protective immunization and not in hypersensitivity. The inciting or shock dose of antigen is a somewhat larger quantity (50-100 times the minimal sensitizing dose in the guinea pig), and is most effective if given intravenously.

The symptoms of anaphylactic shock are peculiarly different in different animals. In the guinea pig shock begins with restlessness, sneezing, rubbing of the nose and increasing respiration. Later the animal has marked difficulty in

breathing, becomes incontinent of urine and feces and may have convulsions. Death is due to respiratory failure. At autopsy the lungs are found distended with air trapped in the alveoli by spasm of the bronchial muscles, and small hemorrhages are seen in many tissues owing to injury of the walls of the small blood vessels. Conversely, the rabbit reacts by the development of convulsions followed by rapid death. The right side of the heart is markedly dilated due to violent spasm of the pulmonary arteries, which prevents the flow of blood through these structures and causes the blood to accumulate in the heart. Other changes observed in anaphylaxis include a loss of the complement and fibrinogen of the blood and a marked activity of the defense cells. Following the anaphylactic reaction, surviving animals are resistant for a time to further shock and are said to be desensitized. They gradually regain sensitivity so that at a later date they may again be susceptible.

In both guinea pigs and rabbits the symptoms and pathology of anaphylaxis are related to the marked contraction of smooth muscle cells. Indeed, the irritability of these cells may be demonstrated outside the body by the **Schultz-Dale reaction**. In this test a small strip of intestine or uterus (both of which are richly supplied with smooth muscle) from a sensitized animal is placed in a bath of Ringer's salt solution and is attached to a recording device. If a small amount of antigen is then added to the Ringer's solution the tissue may be observed to contract vigorously. Suitable animals may be sensitized to a variety of antigens such as pure proteins, blood serum and bacterial antigens. Haptens are not effective sensitizing agents but their injection into a sensitized animal will result in specific anaphylactic shock.

Hypersensitivity may be passively transferred to a normal animal by injection of the blood serum of a sensitized or immunized animal. A few days after injection of the serum the passively recipient animal will develop typical symptoms of shock following the injection of specific antigen.

**The Mechanism of Anaphylactic Shock.** The development of the untoward symptoms of anaphylaxis has been explained by several theories. One of the theories, the humoral theory, ascribed the reaction to the union of antigen and antibody within the blood stream in such a way that a toxic product called **anaphylatoxin** was liberated. Although a number of substances, such as peptone and histamine, produce shock resembling anaphylaxis, the liberation of an anaphylatoxin is not now accepted as explaining the phenomenon. The existing evidence suggests that in anaphylaxis the union of antigen and antibody takes place in association with the body cells. Substances, possibly histamine or histamine-like, which may be directly responsible for contraction of the smooth muscle and the symptoms of shock presumably are liberated in response to the antigen-antibody union. This theory is supported by the remarkable curative action of anti-histamine drugs in hypersensitivity. In the immune animal the antibody circulating in the blood provides protection against this sequence of events, and anaphylaxis is not observed in the highly immunized animal.



**Hypersensitivity in Man.** Human beings rarely develop anaphylaxis but subject to a variety of hypersensitive reactions known as **allergy, idiosyncrasy and atopy.**

Hypersensitivity may develop against many substances and may give rise to a variety of symptoms. The substances found to produce hypersensitivity include foreign serum, such as horse serum, pollens, fungus spores, the dander of various animals, bacteria, foodstuffs, dusts, drugs and an ever-increasing list of chemical compounds. Only some of these substances are protein and hence complete antigens, although others may be antigenic in combination with the **carrier proteins.** The symptoms depend in part upon the portal of entry of the antigen, but the more common ones are the coryza of hay fever, asthma, urticaria or hives, skin eruptions, vomiting, fever and joint pains.

Hypersensitivity in man is naturally acquired, and is presumably the result of previous contact, known or unknown, with a specific antigen. Individuals vary markedly in the ease with which they become sensitized, and several members of the same family are frequently allergic, suffering from hay fever, asthma or some other hypersensitivity. It appears likely that the tendency or predisposition to hypersensitivity is inherited rather than the allergy to a specific antigen. Hypersensitivity, like immunity, is specific and may be passively transferred. Indeed, it behaves and is best regarded as an immune reaction.

**Serum Sickness.** Serum sickness is a form of hypersensitivity observed in a portion of patients treated with antitoxins or antibacterial serums produced from animals. It commonly appears some days after administration of the serum and is characterized by the development of fever, skin eruptions, particularly urticaria, joint pains and at times severe malaise. It may follow the first or subsequent injections of serum and is due to the foreign animal protein rather than to antibodies present in the serum. Rarely a highly sensitive person may develop severe symptoms of anaphylaxis following the injection of serum. The possible occurrence of such symptoms makes it highly important to test for hypersensitivity before beginning treatment with serum. The serum sensitivity test is performed by inoculating a small amount of diluted serum or antitoxin into the skin of the arm or placing a drop of serum into the conjunctival sac. In hypersensitivity a definite inflammation or wheal occurs about the site within 30 minutes. Hypersensitive individuals may be given the needed antiserum if the serum is given very slowly over a long period of time and the patient is observed carefully for any ill effects during its administration. Adrenalin, or antihistaminics, such as benadryl and pyribenzamine, may be used in treatment of shock.

**Allergy to Bacteria.** Hypersensitivity to bacteria or their products, the classical example of which is the positive reaction to tuberculin in man and animals infected with the tubercle bacillus, accompanies a number of bacterial infections. In this and certain other infections local inflammation follows the subcutaneous injection of small amounts of bacterial cell products if the tissues have become sensitive to the organisms present in the body. The anaphylactic reaction may seldom be produced against bacterial antigens.

*Local Hypersensitivity.* In addition to the reactions in which the entire body participates, hypersensitivity may be demonstrated locally. For example, the localized inflammation which appears in a positive tuberculin reaction and various tests for allergy are of this nature. At times hypersensitivity results in the development of local redness, swelling and edema or even severe necrosis and destruction of the body tissue following repeated inoculations of an antigen. The latter reaction, known as **local anaphylaxis** or the **Arthus phenomenon**, does not occur after the first injection but may become increasingly severe after later ones.

**The Significance of Hypersensitivity.** Although they are reactions of the same general type, the consequences of hypersensitivity and immunity are not the same. Hypersensitivity evidently may be harmful; it may also be of little or no significance and rarely, if ever, is it beneficial. Immunity, on the other hand, affords protection against disease agents.



## Pathogenic Microbiology

## 25

## THE STAPHYLOCOCCI

The cluster-forming staphylococci were recognized in cultures by Pasteur (1860) and were isolated from abscesses by Ogston (1881). The first comprehensive study of these organisms was, however, made by Rosenbach (1884) who recognized the now well known *aureus* and *albus* varieties of *Staphylococcus* species. These bacteria are normally present in cultures from the skin, mouth and intestinal and lower genito-urinary tracts of man and animals, and are the organisms most frequently isolated from boils and other abscesses. In addition, they may cause more severe, generalized infections, and certain strains are responsible for outbreaks of food poisoning. The staphylococci are commonly encountered in cultures from the environment as well as in those from the animal body.

**Morphology.** Individual bacteria are usually spherical in shape and differ little in size from one to another culture. They do not form spores or capsules and are not motile. The cells may be arranged singly, in pairs or in irregular grape-like clusters. The tendency to form clusters is characteristic and is the basis for naming these organisms staphylococci. Clusters are best observed in cultures grown on solid media, and short chains of three to four cells are more commonly seen in smears from colonies and cultures in broth.

Staphylococci stain well with ordinary aniline dyes. Young cultures are uniformly gram-positive, whereas old cultures not infrequently are decolorized by the Gram method.

Colonies of staphylococci develop rapidly on nutrient agar medium at ordinary temperatures, and commonly are 1 to 2 millimeters in diameter after 24

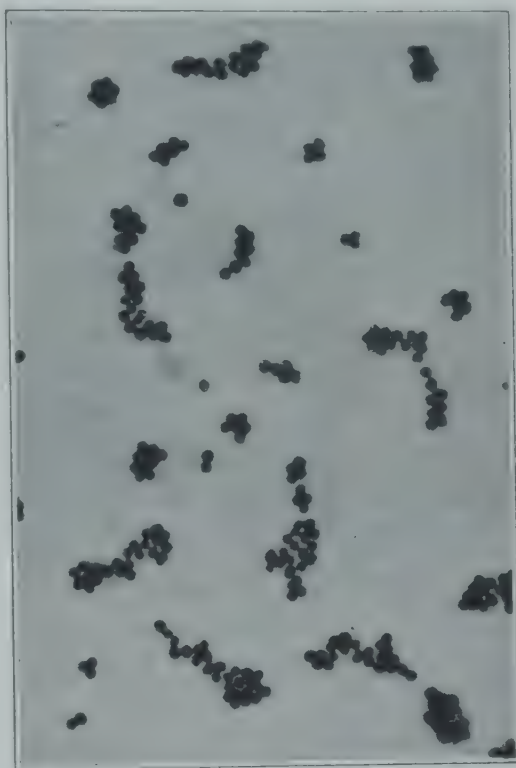


Fig. 141. Photomicrograph of *Staphylococcus albus*. Note the irregular clusters of bacterial cells. (Magnification approximately  $\times 2,000$ .) (Kral.)

hours' incubation. The colonies are moist, smooth, glistening, and slightly elevated with round entire margins. Those of *Staphylococcus albus* are white in color and those of *Staphylococcus aureus* are a golden yellow and those of *Staphylococcus citreus* are a lemon yellow.

**Physiology and Metabolism.** Staphylococci grow best at temperature between  $22^{\circ}$  and  $37^{\circ}$  C, and the usual species develop either in the presence or absence of oxygen. Pigment production, however, occurs only in the presence of oxygen and is best observed in cultures that have grown at lower temperatures.



Fig. 142. Colonies of *Staphylococcus aureus* on blood agar.

These organisms develop well on ordinary nutrient media. Indeed, growth of many strains and, if they are toxigenic, toxin production occur in simple synthetic media containing only a few amino acids, sugar, glucose and the growth factors thiamine and nicotinic acid. Growth may be improved by the addition of biotin and pantothenic acid. Many strains produce hemolysin, and on blood agar medium colonies of such strains are surrounded by a zone of clearing in which the blood cells of the medium have been destroyed.

In general, *aureus* strains are more active metabolically than are *albus* cultures. Acid, but not gas, is commonly produced in broth containing dextrose, lactose, sucrose, maltose and mannitol; litmus milk is acidified and usually coagulated; gelatin is liquefied. Cultures differ greatly in their reactivity, however, so that the biochemical reactions are not a very satisfactory

basis for classification of the staphylococci.

**Pathogenicity.** Staphylococci are the common **pyogenic** or pus-producing organisms for man. They are most often the microorganism cultured from skin pustules, boils (furuncles), the larger carbuncles and abscesses in many parts of the body. They are able to invade the skin through the hair follicles, the openings of the sweat glands and minor injuries. They are often the cause of skin abscess. Although they are of greatest importance as the cause of localized pyogenic infections such as those listed above, staphylococci sometimes invade the blood stream, producing a severe septicemia often with the formation of multiple abscesses in widespread regions of the body; and they may be the cause of endocarditis and meningitis. Both acute hematogenous and chronic recurrent osteomyelitis (bone abscess) in children over two years of age and in adults are commonly due to staphylococcal infection. Staphylococcal osteomyelitis may also follow compound fracture, in which case the organisms may directly invade



used bone. Natural infections occur in animals as well as in man, staphylococci often being isolated from acute and chronic mastitis (abscess of the breast) of cows and from purulent (pus-producing) infections of many animals. In the laboratory, pathogenicity may be demonstrated by the inoculation of experimental animals, particularly the rabbit. *Staphylococcus citreus* is in general non-pathogenic; *Staph. aureus* is more often disease-producing than is *Staph. citreus*. Pathogenicity in 85 to 90 per cent of strains may be correlated with pigment production, the ability to coagulate blood plasma, the fermentation of carbohydrates, liquefaction of gelatin and toxin production.

**Toxin Production.** Filtrates of cultures of toxigenic staphylococci grown under suitable conditions produce several toxic effects, the significance of which was not fully realized until 1928. In that year a number of children given diphtheria toxin-antitoxin for immunization against diphtheria died of acute staphylococcal toxemia. The toxin-antitoxin mixture was found to be contaminated with toxigenic staphylococci. In addition to this lethal or killing effect, which may be demonstrated in experimental animals, staphylococcal toxic filtrates produce **necrosis** or destruction of the skin following intracutaneous inoculation, exert a killing effect on the polymorphonuclear leucocytes of human beings and rabbits, cause **hemolysis** of the red blood cells of a number of different animals and **coagulate** human and rabbit blood plasma. These effects of staphylococcal filtrates have been named, respectively, **lethal toxin**, **dermonecrotxin**, **leucocidin**, **hemolysin** and **coagulase**. It appears that some of the effects may be due to one and the same substance, whereas others are produced by different substances.

Animals injected with toxin may die within a few minutes following inoculation of toxin, or the killing effect may be delayed for many hours or days. The animals develop muscular weakness and paralysis, are unable to coordinate muscular activity and usually experience convulsions before death. In delayed deaths there frequently is vomiting, marked loss in weight, hemorrhages and necrosis of many tissues and circulatory failure. The ability of toxic filtrates to cause necrosis is closely related to the lethal effect.

Staphylococci have been shown to produce two different hemolysins which may be present in toxic filtrates. Some strains produce both types, others produce only a single hemolysin. These hemolysins, which are named **alpha-** and **beta-**hemolysin respectively, affect the blood cells of different animals, are destroyed by heat at different temperatures and are specifically neutralized by immune serum. Alpha-hemolysin is actively hemolytic at 37° C against the red blood cells of mice, sheep, cattle, human beings and several other animals. It is easily destroyed by heat. Immune serum which neutralizes alpha-hemolysin also neutralizes the lethal factor and dermonecrotxin. Beta-hemolysin, on the other hand, causes little hemolysis of rabbit red blood cells, but particularly reacts against those of sheep, cattle and human beings. Hemolysis due to beta-hemolysin is best seen by incubation of tests at 37° C followed by refrigeration, and is often referred to as "cold" hemolysin. Beta-hemolysin is heat resistant, but is destroyed by boiling tem-

peratures. It is neutralized by specific antiserum, but not by antiserum against alpha-lysin. Staphylococcal filtrates may be detoxified by the addition of formalin to produce a toxoid which is antigenic and has been of limited value in the treatment of staphylococcal infections.

Destruction of leucocytes and coagulation of plasma by staphylococci are thought to be due to separate substances from the hemolytic, lethal and dermonecrotic toxins. Both materials are thought to be important in the production of infection by staphylococci. A high proportion of pathogenic staphylococci produce coagulase, which together with hemolysin, pigmentation and fermentation has been used as an index of the ability of cultures to produce disease.

It thus appears that the pathogenicity of the staphylococci is related to toxin production. Some of the properties of culture filtrates (lethal factor, dermonecrotxin and alpha-hemolysin) seem to be related and to possess the properties of true exotoxin.

**Enterotoxin.** Some strains of staphylococci produce a substance, known as enterotoxin, which gives rise to symptoms of food poisoning. Enterotoxin is different from other staphylococcal toxins, is resistant to boiling for some minutes and is poorly antigenic. Typical reactions to enterotoxin begin within 30 minutes to 5 hours following ingestion of toxic material by susceptible human beings. Although man appears to be the most susceptible to enterotoxin, reactions also occur in monkeys, cats and young pigs. Staphylococcal food poisoning is easily recognized when a number of persons are made ill. (See Chapter 10.) Symptoms of the intoxication include vomiting, diarrhea, abdominal cramp, pain, prostration and malaise. Fever is usually absent. The severity of the illness varies from extremely mild to severe, even fatal, reactions and some individuals are not affected by amounts of enterotoxin that are severely toxic to others. The chemical nature of enterotoxin is unknown, but it may be a complex carbohydrate substance produced by the bacteria. Staphylococci that produce enterotoxin cannot be satisfactorily identified except by the production of the characteristic symptoms in suitable experimental animals. The metabolism, pigment production, hemolytic activity and coagulase production are identical with those of nonenterotoxic strains. Fortunately only some staphylococci produce enterotoxin substance.

**Immunology.** Antibodies may be produced against the bacterial cells (agglutinating bacterial serums) or against the toxic products of staphylococci (antitoxins). Although agglutination of the bacterial cells by antibacterial serum occurs, this method has been of little value in the differentiation of staphylococci. By using the precipitin reaction, however, it is possible to separate the majority of strains of staphylococci into pathogenic and nonpathogenic groups. These groups have been found to be related to the presence of type-specific carbohydrate within the cells. About 75 per cent of pathogenic strains may be classified in Type A by the precipitin reaction, whereas most nonpathogenic strains are included in Type B.

Antitoxin which neutralizes the dermonecrotxin, lethal toxin and al-



lysin may be produced in rabbits and horses. This antitoxin has given encouraging but not entirely satisfactory results in the treatment of staphylococcal infections. An international standard antitoxin has been established. The neutralization of staphylococcal toxin by antitoxin appears to be analogous to that of diphtheria toxin by its antitoxin. As noted above, the beta lysin is non-antigenic.

Neither passive immunization with antitoxin, nor active immunization by means of vaccines of toxoid has proved of much value in treatment of staphylococcal infections. Some benefit is obtained in acute generalized disease, but local and chronic infections are little affected. Immunity is short-lived.

**Chemotherapy.** Staphylococcal infections have been treated with a number of drugs of the sulfonamide series. When combined with good medical care, including incision and drainage of abscesses, some favorable results have been reported in acute infections. In general, however, staphylococcal infections respond poorly to these drugs.

A far greater improvement is usually obtained when penicillin is used as the therapeutic agent. This antibiotic substance is highly active against most staphylococci in the test tube and is an effective agent in the treatment of experimental infections in animals. In human infections strikingly beneficial results have been obtained. Acute infections tend to be benefited to a greater extent than chronic ones in which the inflammation is well established. In addition, a number of staphylococci are resistant to the action of penicillin and others become resistant during contact with it. Nevertheless, treatment of staphylococcal infections with penicillin is the method of choice at the present time.

Aureomycin also appears to be effective against staphylococci.

## 26

### THE STREPTOCOCCI

Chain-forming cocci, that is, streptococci, have been recognized since the early days of bacteriology. Indeed, they were associated with the disease erysipelas by Fehleisen in 1883, and a year later were named by Rosenow. As a group the streptococci are among the most important agents of disease in man and animals.



Fig. 143. Photomicrograph of stained smear of broth culture of beta-hemolytic streptococci. Note the long chains of the bacteria. (Magnification approximately  $\times 2,000$ .)

In addition, members of the group are normally present in the mouth and throat and the intestinal contents, and may be isolated from milk and other dairy products. The streptococci are primarily parasites of man and animals and are not commonly found in the soil or uncontaminated water.

The streptococci are usually described as round or ovoid cells, typically Gram positive and arranged in chains of varying length, sometimes in pairs. They do not form spores and are nonmotile. Some species form capsules. Most species are aerobic or facultatively anaerobic. They ferment carbohydrates with the production of acid.

**Morphology and Growth.** The individual streptococcal cells are typically spherical cocci, although some strains form elongated cells and some have a tendency to be rather flattened or bean-shaped. In young cultures they typically retain the Gram stain and are easily colored by the usual bacteriological dyes. Older cultures

tend to lose the ability to retain the Gram stain. The streptococci are not acid-fast.

The length of the chains of streptococci is a highly variable feature; some form short chains of four to eight cells, whereas others may grow in chains of twenty, thirty or more cocci. In general, chain formation is more marked in the hemolytic and pathogenic group than in the less virulent species, and is more



of cultures in liquid than on solid media. In some cultures the chains are to be made up of pairs so that the basic arrangement may be diplococcal. pneumococci, which are usually included in the tribe of the streptococci, are usually arranged as pairs (diplococci) of elongated lanceolate-shaped cells. pneumococci will be discussed in the succeeding chapter.

The colonies of streptococci on solid media tend to be small, from 0.5 to 1.0 in diameter, discrete, convex and semitranslucent. The colonies may be smooth and entire, rough and granular or intermediate. The intermediate type colony is commonly formed by virulent strains. Pigmentation is not characteristic of the streptococci. In broth the growth tends to be granular and to settle onto the bottom and sides of the tube. Some strains, however, give a stringy growth in broth and others, which grow in short chains, produce a diffuse clouding of the medium. Some species develop poorly on ordinary nutrient media and require, at least initially, infusion media or media containing blood, serum, ascitic fluid or similar enriching substances. As a group they are rather complicated media for growth, although a few have recently been grown on relatively simple, chemically defined media. The known growth requirements are not uniform but various members of the group require different factors.

**Physiology and Metabolism.** The streptococci possess highly varied metabolic activities which together with their immunology provide a basis for their division into several groups. Their reactions are discussed here as individual reactions and the correlation of the activities of individual species or groups is dealt with in the section dealing with classification.

The majority of species grow well either in the presence of air (oxygen) or in its absence. Some species, however, are strictly anaerobic and others develop only if the oxygen tension is reduced. The latter groups will be discussed with nonsporulating anaerobic bacteria.

Usually, development is good at either room or body temperature (22° to 37° C), although the groups differ in ability to grow at excessively high or low temperatures. Indeed, the ability to grow at 10° C and at 45° C is an important differential characteristic. The pyogenic cocci usually fail to develop at these temperatures and other groups are variable in this respect. The pyogenic cocci are generally killed by heating at 60° C for 30 minutes, whereas the streptococci and some members of the other groups usually resist this treatment. Other metabolic properties are equally variable, so that the ability to grow in broth containing 6.5 per cent sodium chloride, to reduce methylene blue, to produce nitroprusside and other dyes to the corresponding colorless compounds, to produce ammonia from peptone, to hydrolyze sodium hippurate, starch and esculin, and to ferment numerous carbohydrates are important differential characteristics. The medium is usually acidified and commonly coagulated. Some cultures are proteolytic and they liquefy gelatin and digest the casein of milk. The majority, however, do not possess this property.

**Reactions on Blood.** The streptococci may be divided into groups according to their reactions on blood agar. These reactions are illustrated in Fig. 144.

Observation of the changes in blood are best made by using 5 per cent blood agar pour plates, but they may be made by using the usual blood streak plates.

The alpha ( $\alpha$ ), green-producing or viridans streptococci produce a greenish zone of partial hemolysis surrounding the colony on blood agar. Following continued incubation at body temperature or more often after refrigeration overnight, a narrow clear zone of hemolysis may be observed surround-

the green zone. Repeated incubation and refrigeration result in the development of multiple concentric rings of green discoloration and clearing. Under the microscope the red blood cells in the medium are found to be only partially destroyed.

The beta ( $\beta$ ) or hemolytic streptococci produce a sharply defined broad zone of complete clearing of the medium about the colony in which there is complete destruction of the red blood cells.

The gamma ( $\gamma$ ) or indifferent streptococci grow in blood agar without producing any visible change in the medium.

Some cultures do not correspond exactly to the above descriptions. Thus some cultures, designated alpha prime ( $\alpha'$ ) produce partial clearing of the medium without greening, and still others produce some greenish discoloration without giving the typical, multiple zone, alpha reaction.

The ability of streptococci to cause beta-type hemolysis is correlated in a general way with the production of hemolysin in liquid media, that is, streptolysin, and with pathogenicity. There are two types of streptolysin, one of which (streptolysin O) is sensitive to oxygen and is readily inactivated in the presence of air, whereas

the other (streptolysin S) is active either aerobically or anaerobically. Some alpha strains in addition to the majority of true beta strains produce appreciable amounts of soluble hemolysin, and when incubated anaerobically on blood agar may cause a beta-type reaction. The greenish discoloration produced by alpha-type cultures was formerly thought to be due to methemoglobin formation. That this is not the case is, however, generally accepted at the present time, and the greenish color is explained by the formation of a product of hemoglobin destruction, possibly by reduction, which as yet is an incompletely identified, iron-containing substance.

**Fibrinolysin.** In addition to the effects on the red blood cells, certain streptococci produce fibrinolysin (see Chapter 22). This substance, which produces the liquefaction of clotted human blood plasma and of purified fibrin

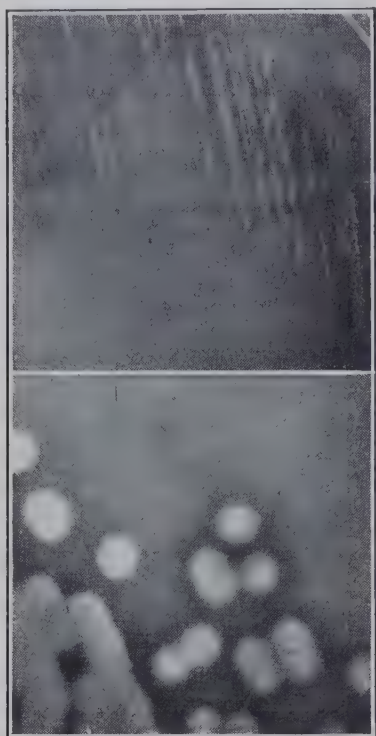


Fig. 144. Colonies of alpha (upper) and beta (lower) type streptococci on blood agar. Note the changes in blood medium surrounding the colonies ( $\times 2$ ).



duced only by the hemolytic streptococci pathogenic for man (particularly of Group A). Its production is thus correlated with pathogenicity.

**The Antigenic Structure of the Streptococci.** Differentiation of streptococci by immunological methods, particularly agglutination and precipitin reactions, has aided greatly in the study of human disease and in the recognition of pathogenic members of the group. The antigenic structure of hemolytic streptococci isolated from human disease processes has, of course, received the greatest attention. Within this group Griffith has been able to recognize 27 different types by means of the agglutination reaction, which are now referred to as Griffith types. By means of the precipitin reaction using extracts of the bacterial cells, Lancefield first separated the hemolytic streptococci into broad groups and then defined type-specific antigens. The Lancefield groups are determined by the presence of a carbohydrate antigen known as the "C" substance, which is different for each group. At present nine groups of hemolytic streptococci are recognized as follows:

- GROUP A — Hemolytic streptococci pathogenic for man.
- GROUP B — Animal strains, particularly those of bovine mastitis.
- GROUP C — Hemolytic streptococci from human and animal sources.
- GROUP D — Hemolytic and nonhemolytic enterococci.
- GROUP E — Hemolytic streptococci isolated from milk and animal sources.
- GROUP F — Minute hemolytic streptococci from normal and diseased human beings.
- GROUP G, H and K — Hemolytic streptococci found in normal mucous membranes and skin.

Of these organisms only those of Group A have a clearly established pathogenicity for man, although members of Groups C, D, F, G and H are isolated from human beings and some may be associated with illness.

The differentiation of the Griffith types of Group A streptococci has been found to depend upon the presence of two antigens, the "M" and "T" substances. The "M" substance is type-specific, is found in virulent strains and is destroyed by proteolytic enzymes. The "T" substance is independent of the "M" substance, is commonly found in both virulent and avirulent strains and may be found in more than one type. When injected into animals these antigens stimulate the formation of different antibodies.

**Classification of Streptococci.** The recognition of species of streptococci is a difficult task, and there is not general agreement on the classification. However, the separation of these organisms into broad groups and the typing of hemolytic strains is now widely accepted. The comprehensive classification of streptococci of man is based upon both physiological and immunological characteristics and is here followed in general in this book.

**The Pyogenic Streptococci.** These are the hemolytic or beta-type streptococci. They produce soluble hemolysins, cause the beta reaction on blood agar and are relatively susceptible to alkali and salt. Further differentiation is dependent upon recognition of the Lancefield groups and, in Group A, the Griffith types. Fermentation reactions are of some value in the classification. As has

been indicated, the human pathogens are usually members of Group A, and in general designated as *Streptococcus pyogenes*, *Str. hemolyticus* or beta streptococci. The human pathogens also have often been named as specific disease agents, such as *Str. scarlatinae* isolated from scarlet fever, *Str. erysipelatis* from erysipelas and *Str. epidemicus* from septic sore throat. At present they are better differentiated as Griffith types than by the diseases resulting from infection with them, since several types may give rise to the same disease (pathogenicity, below). Several organisms pathogenic for animals also are included in the pyogenic streptococci; *Str. mastitidis* (Group B) is commonly isolated from bovine mastitis; *Str. equi* (Group C) is frequently isolated from strangles, a disease of horses.

**The Viridans Group.** The members of this group characteristically produce an alpha-type reaction on blood agar. They grow at high but not at low temperatures and are susceptible to salt, alkali and dyes. Both human and animal types are included in this group, but those of medical importance are the *salivarius* and *Str. mitis*. These organisms are commonly found in the nose and throat and may be isolated from the intestinal contents and from the female genital tract. As agents of disease they are the streptococci most commonly associated with endocarditis. It should be pointed out that members of the viridans group are not the only streptococci that produce greening or alpha reactions on blood, but that members of the lactic group and the enterococci may produce similar changes in blood. The *Str. viridans* of the clinician may be a member of any one of these groups unless properly separated from the other groups by cultural and immunological reactions. Immunological reactions have been of little value in separating members within the viridans group.

**The Lactic Streptococci.** The lactic streptococci are not important human pathogens. They are, however, used in biological assays for nutritional substances and in the dairy industry for the manufacture of cheese and artificial butter. Lactic streptococci predominate in sour milk. Members of the group are highly acidogenic, grow at low temperatures, are resistant to heating and are strong reducing agents.

**The Enterococci.** The enterococci are commonly found in the intestinal contents of man and animals. The group is distinguished by the resistance of the organisms to heating, alkali, salt and bile, strong reducing action and the high acidity produced in carbohydrate media. The enterococci, both hemolytic and nonhemolytic, have been found to belong to Lancefield Group D. Although they are commonly inhabitants of the intestinal tract, the enterococci, particularly *Str. faecalis*, may be pathogenic. This organism has been recovered from the blood in endocarditis and from infections in various locations. It is important as the causative agent of infections of the urinary bladder.

**Pathogenicity.** The streptococci may cause infection as primary invaders or as opportunists in many locations in the body, the list of diseases including local or pyogenic infections, septicemia, meningitis, pneumonia, infection of heart valves (endocarditis), puerperal infection, erysipelas, sore throat, sc



and food poisoning. The virulence of different kinds of streptococci is very different, that of the human pyogenic or Group A streptococci being the greatest for man. Given the opportunity, however, members of other groups, particularly those commonly present on the mucous membranes of the mouth, throat or intestinal tract, may cause severe and fatal infection.

Although a variety of clinical diseases due to infection with hemolytic streptococci is recognized, it must be remembered that the causative organisms are usually members of Lancefield Group A, and that they characteristically invade mucous membranes. There appears to be no specific correlation between the biological type of the organism and the ability to cause a particular clinical infection, although some types are encountered more frequently than others. Furthermore, a number of streptococcal throat infections are commonly observed during epidemics of scarlet fever, and streptococci isolated from scarlet fever patients will reproduce the disease in certain individuals but will cause pharyngitis without the skin eruption in others. There is, therefore, a tendency to regard outbreaks of disease as **streptococcosis** in recognition of the common etiology.

Streptococci pathogenic for man are virulent for a number of animals, including rabbits and guinea pigs, and may occasionally naturally infect domestic animals. Of far greater importance in veterinary infections are the animal pathogens included in the higher Lancefield groups, such as *Str. mastitidis* (*Str. lactiae*) and *Str. equi*. In animals either localized infections or septicemias may be produced.

**Toxins.** The virulence of pyogenic streptococci, although not completely defined, is in large part related to the formation of soluble toxic substances. Filtrates of cultures of these organisms have been shown to contain hemolysins, streptolysin, streptokinase, fibrinolysin, spreading factor and an erythrogenic or scarlatinal toxin. The activity of a number of these substances has been discussed in the chapter dealing with bacterial virulence and will not be repeated here. Some have been demonstrated in the body fluids of persons suffering from streptococcal infection, and their activity undoubtedly aids the streptococci in invasion and destruction of the body tissues.

**Erythrogenic Toxin.** The erythrogenic or scarlatinal toxin is produced by certain strains of Group A streptococci, but its formation is not peculiar to any one type within the group. The toxin accounts in large part for the general character of scarlet fever, for the skin eruption and redness, the aching and malaise. The scarlatinal toxin is an exotoxin which may be demonstrated in cell-free filtrates of positive cultures. It may be inactivated by heating, and it is antigenic, both stimulating formation of and being neutralized by specific antitoxin. Injection of erythrogenic toxin into the skin of susceptible individuals causes the development of an area of redness or erythema at the site and, in large quantities, may produce a more generalized reaction resembling scarlatina. The chemistry of erythrogenic toxin is relatively unknown, but it appears to be separate from other streptococcal toxins and unrelated to the type-specific antigens.

**The Dick Reaction.** The erythematous reaction produced by injecting erythrogenic toxin into susceptible persons is used as a measure of susceptibility to scarlet fever. In performance of the test a dilution of streptococcal filtrate containing one Skin Test Dose (STD) of toxin is injected into the skin of the forearm. In a positive reaction, indicating susceptibility, an area of redness appears at the site within 24 hours. The Skin Test Dose is standardized to produce an area of redness 1 cm. in diameter in susceptible persons. Failure of a reaction to appear or the presence of a reaction to both toxin and broth controls indicates immunity; that is, the toxin is neutralized by antitoxin. The reaction in scarlet fever is analogous to the Schick reaction in diphtheria.

**Scarlet Fever (Scarlatina).** Streptococci have long been associated with scarlet fever as the causative agent. However, the proof of the relationship was not obtained until 1923 when the Dicks succeeded in reproducing the disease in human volunteers with a culture of hemolytic streptococci isolated from a scarlet fever patient. Later the same authors demonstrated the development of the erythematous rash following the injection of culture filtrates and developed the **Dick test** for detecting susceptibility. Furthermore, neutralization of erythrogenic toxin by immune serum and antitoxin, together with the demonstration that such serum when injected into the area of skin rash will cause a disappearance or blanching of the rash (**the Schultz-Charlton reaction**) provided supporting evidence of the causation of scarlet fever by hemolytic streptococci.

Scarlet fever in its typical forms is an acute infection of the nasopharynx by hemolytic streptococci, which is associated with the development of a red flush to the skin of the entire body and a fine red eruption of the skin. It commonly begins one to two days after acquisition of the organisms and lasts about two weeks. During the acute stages, cultures from the throat plated on blood agar media reveal large numbers of hemolytic streptococci which may be classified immunologically as any one of several types of Lancefield Group A. The disease may, indeed, be diagnosed by the finding of such streptococci in cultures from persons having clinical findings of the disease. Diagnosis is aided by demonstration of the Schultz-Charlton blanching reaction. Scarlet fever occurs chiefly in persons who are positive reactors to the Dick test, although a small proportion of patients are found to give negative reactions before as well as after illness. Convalescence is associated with a rise in antitoxin titer of the blood and hence with development of a negative Dick reaction. Antifibrinolysin may be demonstrated in convalescent serum.

**Prophylactic Immunization.** Immunity to scarlet fever, as indicated by the Dick test, is frequent among infants during the early months of life as passive immunity, and among adults as actively acquired immunity. The resistance in the latter group may have been acquired as the result of either an active course of the disease or from atypical and inapparent infection with streptococci. Preschool and school age children react positively to the Dick test in a high proportion of cases.



Following their demonstration of erythrogenic toxin, the Dicks developed immunizing procedure using small doses of the toxin. The usual practice is five inoculations increasing from 500 to 100,000 STD of toxin in a series of five or more graduated weekly doses. The inoculations result in conversion of a positive to a negative Dick reaction in over 95 per cent of persons. In group tests inoculations have resulted in a reduction of the number and severity of the outbreaks of clinical scarlet fever. However, the reactions to toxin may be generalized and severe, and the immunity protects only against the effects of erythrogenic toxin and not against streptococcal infection. Prophylactic immunization against scarlet fever is not generally practiced and, because of the relative mildness of present-day scarlet fever and the newer methods of treatment, it is of less value than in the past.

**Scarlet Fever Antitoxin.** As has already been mentioned, antitoxin which neutralizes the erythrogenic toxin is present in the blood of persons convalescent from scarlet fever. Antitoxin may also be produced by the immunization of animals. Either convalescent human serum or equine antitoxin may be used in the treatment of scarlet fever. The unit of antitoxin is that amount of serum which will neutralize 50 STD of toxin. A dosage of 9000 units of antitoxin is used in the treatment of scarlet fever. Antitoxin treatment commonly results in relief of the skin eruption and symptoms attributable to the erythrogenic toxin, and patients who have received antitoxin reportedly have fewer complications from the infection. However, the sulfonamide drugs or the antibiotic penicillin are most often used in treatment at the present time.

**Erysipelas.** Erysipelas is an infection of the skin characterized by a rapidly spreading, intensely red and sharply bordered inflammation. Although hemolytic streptococci may be cultured from the lesions by appropriate methods, the diagnosis is usually a clinical one. The mechanisms of disease production are obscure, but the disease is apt to recur in susceptible persons, and produces a localized, local tissue-immunity. Erysipelas was in former years a very serious disease, but with the advent of present-day chemotherapy its effects have been rendered less formidable.

**Septicemia.** The tendency of hemolytic streptococci to invade generally the deeper tissues from an initial site of infection is well known. Thus spread through lymphatic and blood vascular systems is not infrequently witnessed during infections of many regions of the body. Septicemia commonly occurs in puerperal fever and may be present in streptococcal pneumonia or as a complication of scarlet fever, wound infections and endocarditis. Nonhemolytic streptococci may invade the deeper tissues and blood from the mucous membranes or from focal infections, such as inflammation of the gums or tonsils. Septicemia is periodically present in endocarditis.

**Puerperal Fever.** The infectious nature and often epidemic character of puerperal or childbed fever was early recognized by Oliver Wendell Holmes in this country and by Semmelweis in Austria. The disease is now known to be caused by infection of the uterus, the surrounding tissues, the peritoneal cavity

and the blood with any of a number of organisms. However, the hemolytic streptococci are one of the most important causes of severe and septic puerperal fever. Infection of the genital tract with *Str. pyogenes* is usually introduced from elsewhere (exogenous infection), possible sources of infection being the patient herself, other patients and personnel who harbor virulent organisms. Prevention of infection therefore assumes a prominent role in the care of puerperal patients. Cultures from the uterine cavity and, in septicemia, the blood commonly reveal the causative organisms. Nonhemolytic and anaerobic streptococci also are often isolated from patients with puerperal infection, either in pure culture or in association with other microorganisms.

**Subacute Bacterial Endocarditis.** Bacterial infection of the heart, particularly when these structures have been previously damaged, is a serious and fatal disease. Although a number of bacteria may opportunistically infect the heart valves, the most common causative organisms are the nonhemolytic streptococci. Actually several species, usually members of the viridans or *Diphtheria* coccus groups of Sherman, may be the causative agents. These are usually nonpathogenic producing organisms and are commonly not further identified. Endocarditis is particularly resistant to treatment and until very recent years was uniformly fatal. However, encouraging results have been obtained with antibiotic therapy.

**Rheumatic Fever.** Rheumatic fever and the associated rheumatic disease is presently the leading cause of death of school-age children, and among the leading causes of disability and death throughout childhood and adult life. The causes of rheumatic fever remain obscure. Some investigators have attributed it to infection with a filtrable virus, whereas others have regarded it as one manifestation of streptococcal infection. The evidence is largely circumstantial and is based upon the frequency with which an attack of rheumatic fever follows acute hemolytic streptococcal infection, the frequent finding of antibodies against streptococci in the blood of rheumatic fever patients and the similar geographical distribution of rheumatic fever and streptococcal infection. The evidence, although circumstantial, is impressive, and the view that rheumatic fever is a manifestation, possibly allergic, of streptococcal infection is well held. Recent evidence strongly supports the allergic theory and suggests the possibility of multiple sensitizing agents. Hemolytic streptococci may well be the most important of these agents.

**Epidemiology of Human Streptococcal Infection.** Nonhemolytic (alpha and gamma type) streptococci are universally present on the mucous membranes of the mouth, pharynx and lower intestinal tract of man and animals. Hemolytic streptococci are found less frequently in the normal body. The latter organisms are more frequently found in the winter than in the summer months, and are particularly prevalent among the contacts and associates of patients with scarlet fever or other streptococcal infection. It is commonly estimated that about 10 per cent of people have hemolytic streptococci in culture from the throat, a fact which is consistent with the frequency of disease caused by them.



these organisms and which justifies the frequent cultures of the noses and throats of personnel caring for obstetrical and pediatric patients. Scarlet fever in the past was one of the most devastating diseases of childhood, and it continues to be one of the most frequent and important, if now less common, infections. Recent figures (1938-1942) indicate a median number of over 1000 cases per year and a mortality of over 3.0 per 10,000 population. Streptococcal sore throat, erysipelas and puerperal fever account for an additional number of illnesses, and other hemolytic streptococcal infections are frequent.

Hemolytic streptococci are usually transmitted from person to person by more or less direct contact, by droplet and air-borne infection (see Chapter 44). However, milk may be contaminated with pathogenic streptococci either from infection of the bovine udder or directly from milk handlers. Furthermore, food handlers may contaminate food during its preparation. Streptococci are able to multiply in milk and food which are improperly stored, so that a small initial inoculum may become a dense culture in a few hours.

**Chemotherapy.** Chemotherapy has largely replaced serum treatment of streptococcal infections. The hemolytic streptococci pathogenic for man are sensitive to the sulfonamide drugs and infections with these organisms were among the first successfully treated by these agents. Indeed, sulfonamide therapy was the first satisfactory method for the treatment of many infections caused by streptococci, and its use resulted in a marked reduction in the number of deaths and in the severity of these diseases.

In addition, streptococci, with the major exception of the enterococci, are sensitive to penicillin, a fact of particular importance in the treatment of rheumatic carditis, although the antibiotic is now commonly used in treatment of all streptococcal infections. The sensitivity of anaerobic streptococci has not been fully established. Aureomycin is also active against streptococci.

There is some evidence that the improvement in response to the newer methods of therapy may be so rapid that the patient may not develop a solid immunity against subsequent infection. Should the lack of immunity following infection become a frequent occurrence, the incidence of multiple attacks would have all likelihood be considerably increased.

# 27

## THE PNEUMOCOCCI

The pneumococci are closely related to the streptococci in their morphological and cultural characteristics. Indeed, they may be classified as *Streptococcus pneumoniae*, although more commonly they are placed in a separate genus, *Diplococcus*. The pneumococci are considered to represent a single species, *Diplococcus pneumoniae*, the subdivisions being referred to as immunological types. These organisms were early described as the causative agent of pneumonia by Fraenckel and Weichselbaum. Their importance is due largely to their particular ability to invade the lungs.

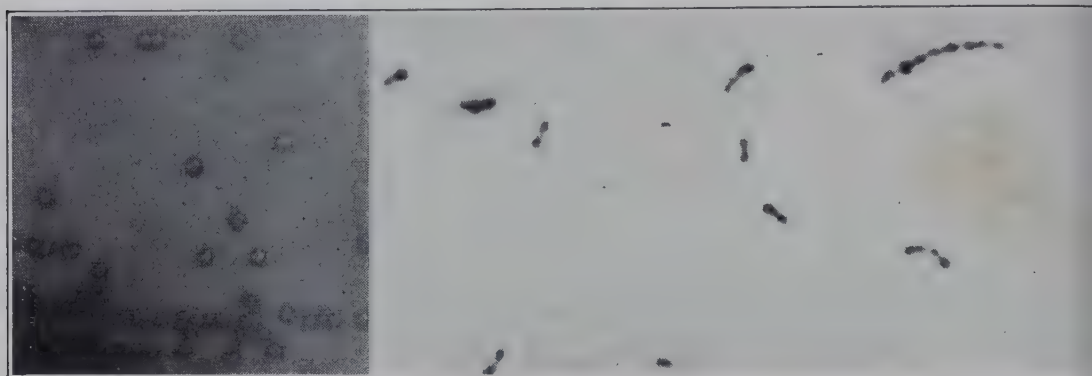


Fig. 145. (Left) Colonies on blood agar and (right) photomicrograph of pneumococci. (Magnification approximately  $\times 1400$ .) The colonies are surrounded by a zone of partial destruction of the blood similar to that produced by alpha-type streptococci. Note the elongated lancet shape of the pneumococcus cells.

**Morphology.** As the genus name *Diplococcus* implies, the pneumococci cells are typically arranged in pairs. The individual cells tend to be elongated or lancet-shaped and the cocci are flattened on adjacent surfaces (Fig. 145). Usually seen, the bacteria are surrounded by a capsule of amorphous, gummy, gelatinous material, which is unstained by the usual dyes. Not infrequently chains of four or more cells are observed. The pneumococci are gram-positive in young cultures, are nonmotile and do not form spores. In older cultures gram-negative cells are commonly seen.

Colonies of pneumococci on blood agar medium are round, translucent, smooth, entire, flat-surfaced or rather droplet-like in appearance. They are about 0.5 to 1.0 mm. in diameter and are surrounded by a zone of greenish alpha



lysis. The colonies of pneumococci, therefore, may not be differentiated with certainty from those of the alpha-type streptococci. In infusion or serum broth organisms grow abundantly, giving a uniform turbidity to the medium.

**Physiology and Metabolism.** The pneumococci grow rapidly and well in enriched infusion or serum-containing media at temperatures near the body temperature, but they do not develop at high or low temperatures. They grow poorly in an excessively acid or alkaline medium. Cultures of pneumococci die rapidly during prolonged incubation, a fact presumably related to their inability to produce the enzyme catalase, to the consequent accumulation of hydrogen peroxide in old cultures, and to the rapid autolysis of cells. The pneumococci are, indeed, relatively delicate bacteria. Of particular importance for their identification is the dissolution of pneumococcal cells by bile, bile salts and certain detergents. Usually the **bile solubility** test is conducted by adding 0.1 ml. of a 10 per cent solution of sodium taurocholate to 1.0 ml. of a young pneumococcus culture. Within 30 minutes the pneumococcus cells are completely lysed or lysed, whereas streptococci are unaffected by similar treatment.

Pneumococci produce acid but no gas from several sugars, including lactose, sucrose and, in contradistinction to the streptococci, inulin. Milk is acidified and can be coagulated. Gelatin, tryptophane and nitrates are not attacked.

Growth of a number of pneumococcus types has been obtained in a hydrogelatin or an amino-acid medium containing glucose, salts and added growth factors. Pantothenic acid, biotin, nicotinic acid, choline and a reducing agent, such as thioglycollic acid, appear to be essential for growth, although requirements of the various types are not uniform.

**Variation.** As in the case of other bacteria, the pneumococci undergo smooth to rough ( $S \rightarrow R$ ) variation. The smooth colony type described above is characteristic of encapsulated, type-specific and fully virulent pneumococci and is absent in nonencapsulated variants by a rough colony form. The absence of type specificity and virulence in rough strains of pneumococci is also associated with the loss of ability to produce capsules. Smooth to rough variation may be induced in the laboratory by cultivating smooth encapsulated strains in broth containing homologous immune serum and may occur in cultures maintained for long periods of time on artificial media. Furthermore, rough variants may be induced during the course of naturally occurring pneumococcal infections. Reversion, that is, rough to smooth variation, may be brought about by the cultivation of rough strains together with anti-R immune serum, heat-killed encapsulated cells or extracts thereof (see below) and by animal passage.

**Chemistry of the Pneumococcus Cell.** Study of the chemistry of the pneumococcus cell has contributed greatly to an understanding of both the mechanisms of development and the treatment of pneumococcal infection and to the theory of immunology and bacterial genetics. It has been possible by chemical methods to separate and to identify in part the constituents of the capsules and the cell substance. Thus the capsular material, known as the soluble capsular substance or SSS because each pneumococcus type possesses a chemically

and immunologically different capsule, is now known to be a complex carbohydrate or polysaccharide. This polysaccharide is made up of chemical combinations of simpler carbohydrates, which differ in composition and/or combinations of capsular materials from different types of pneumococci. Studies of the SSS of Types I, II and III pneumococci have shown, for example, that simple sugars such as glucose, and sugar acids, such as galacturonic and glucuronic acids, are important units of the polysaccharides of one or more of these types. The pneumococcal SSS is immunologically a haptene and as first isolated was found to be incapable of inducing specific antibody formation except in combination with the cellular antigens, but capable alone of reacting with such antiserum. However, the polysaccharides of Types I and II have since been shown to be complete antigens capable of stimulating the formation of antibody. That relationships exist between the SSS of different pneumococcal types is shown by cross-reactions between different types, a point which is clarified in Chapter 23.

The cell substance itself appears to be the same in all pneumococcus types and is composed of proteins, nucleic acid, lipoidal materials and carbohydrates. Antigenically, the somatic or cellular antigens from the various types of pneumococci and from nonencapsulated R variants are the same, since antisera prepared against the cells from one will react equally well with the cellular antigens of the others.

Of particular interest and biological importance are the studies of the transmutation or conversion of the cells from one type of pneumococcus into another. Some years ago it was observed that the injection into animals of avirulent cells derived from one pneumococcus type together with heat-killed cells of a second, different type encapsulated culture often led to death of the animal from infection with living organisms of the second type. The living R cells had assumed the virulence and specificity of a type of pneumococcus different from the parent culture. Many experiments have now revealed that such transformation of types may be produced in the test tube under carefully controlled conditions of growth not only by killed, type-specific cells but also by means of extracts of these cells. In extensive chemical fractionation studies the active principle has been found to reside in the purified desoxyribonucleic acid fraction of the cell substance. Also, the transforming activity is lost during treatment of the extracts which destroy desoxyribonucleic acid. Neither other portions of the cell nor the SSS have been found capable of inducing the transformation. Thus, the cell substances of different pneumococcus types possess different biological activity in the determination of type specificity, although immunologically the somatic antigens appear to be identical. The cells after transmutation possess all of the properties of the new type, a fact of great significance. Natural occurring transformation of types is unknown.

**The Pneumococcus Types.** It was early recognized that antiserum against one pneumococcus strain failed to protect animals against antigenically different pneumococci (Neufeld and Handel), and that pneumococci from human pneumonia could be divided into three types, Types I, II, and III, and an immunologically



heterogeneous Group IV (Dochez and Avery). Later extensive study of pneumococci from human sources by Cooper (1918) resulted in the recognition of an additional 20, giving a total of 32 types (Types I to XXXII). However, all pneumococci may not be classified within these groups, and recent studies recognize a total of 75 antigenically different types. As indicated above, the type specificity of pneumococci is determined by capsular haptene, the SSS, which reacts immunologically with type-specific

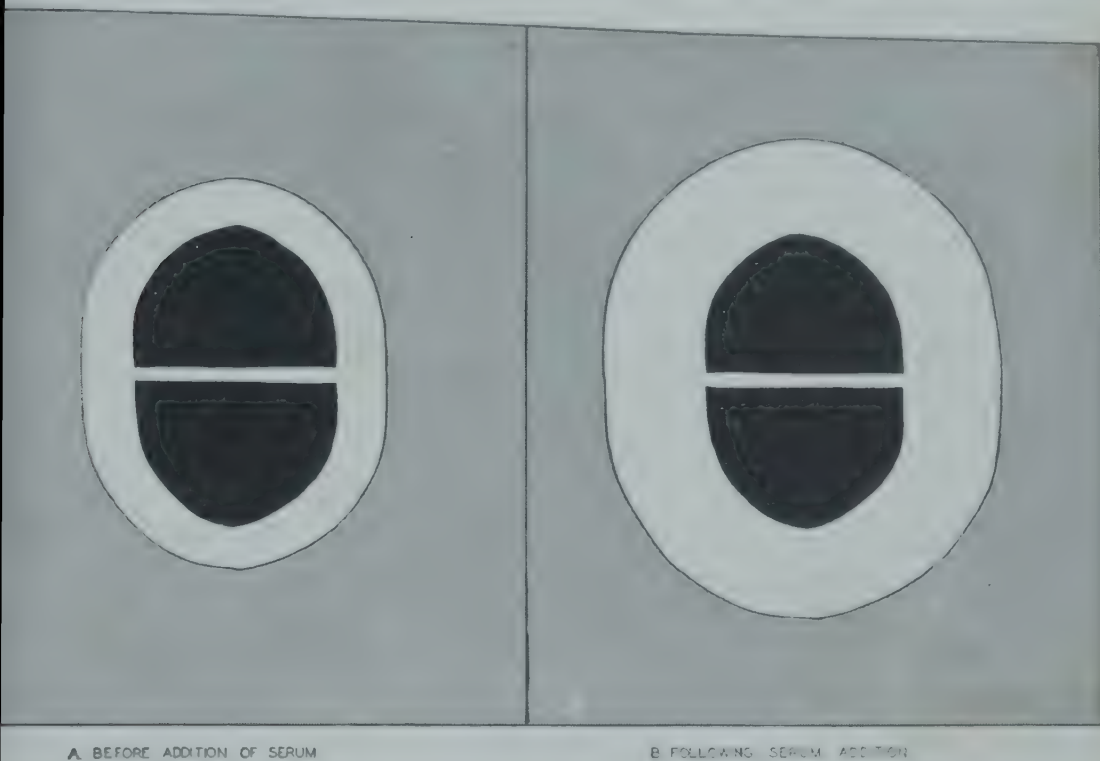


Fig. 146. Diagram of the Neufeld capsular swelling (Quellung) reaction. In the presence of specific immune serum the capsule of the pneumococcus and similar forms becomes greatly enlarged.

serum. Any of several immunological reactions may be used for pneumococcus typing, including the agglutination test (using as antigen the encapsulated whole bacterium), the precipitation test (performed with antigens composed of body fluids or culture extracts containing the SSS) and the Neufeld "Quellung" reaction. The **Quellung or capsular-swelling test** (Fig. 146) is performed by adding a drop of sputum, other body fluids containing pneumococci or a young pneumococcus culture to a drop of typing serum. The materials are mixed on a glass slide, usually with a drop of methylene blue stain to aid visibility, a coverslip is applied, and the preparation is observed under the microscope. When pneumococci are mixed with antiserum of the homologous type, the capsule becomes much enlarged or swollen. In typing pneumococci from patients with pneumonia, the Neufeld reaction is applied directly to the sputum if that material contains sufficient organisms. Otherwise, sputum may be washed in saline and the washings injected into the peritoneal cavity of a mouse. Within

a few hours (3 or more) the Neufeld reaction or other typing procedure may be performed on the peritoneal washings from the mouse.

An optional method, or one that may be used in conjunction with the injection method, is to cultivate the organisms from sputum in an enriched or serum broth for 4 to 8 hours before typing. The rapid growth of the pneumococci results in an abundant culture within this time. The rapid typing of pneumococci from patients with pneumonia is important to serum treatment of the disease, since type-specific antiserum cannot be administered without knowledge. The labor of typing is greatly reduced by first using grouping sera against several related types or against convenient groups and then identifying the organisms with individual, type-specific serums. In the case of young children who do not produce sputum, pneumococci may often be recovered from throat cultures or stomach washings. Also, blood cultures should be taken; they usually yield positive results. The isolation of colonies is best accomplished by cultivation of specimens directly on blood agar plates, or it may be done by simultaneously cultivating mouse peritoneal exudates or enrichment cultures.

Immunological cross-reactions occur between a number of the pneumococcal types, undoubtedly reflecting chemical similarity in the capsular substance. Thus, the cells of Type II react with antiserum against Types II, V, X, XI, and XX; those of Type III with antiserum against Types III, VIII, and XIX; Type VII cells with antiserum against Types II, VII, XVIII and X; Type X cells with Type X and XIII antiserum, etc. Furthermore, close relationships exist between some strains which are not identical, so that subtypes such as VIIA and XVA, have been recognized. These relationships do not validate the type specificity.

**Pathogenicity.** The pneumococci are most important as the causal agents of human primary lobar pneumonia. However, pneumococci are also commonly found in infections of the upper respiratory tract and its communicating passages, including the middle ear (otitis media) and the air sinuses, such as the mastoid cells, and they are among the chief causes of meningitis. Infections of other mucous membranes and of the deeper tissues are occasionally encountered, and septicemias and peritonitis are sometimes caused by pneumococci. The pneumococci are highly pathogenic for certain laboratory animals, particularly mice and rabbits, and a disease closely similar to pneumonia in man may be produced experimentally in monkeys and in dogs by intratracheal or intrapulmonary inoculation of pneumococci, preferably in a mucin suspension. Studies of these infections have added greatly to knowledge of the disease process in man.

Virulence is a property of smooth pneumococci, is dependent upon the presence of the capsule and is absent from rough, nonencapsulated cultures. Exotoxin is not formed. However, soluble hemolysin, which appears to be somewhat antigenic and associated with pneumococcal proteins, leucocidin and a necrotizing substance are formed. The role of the latter substances in pathogenicity is uncertain. Extracts of pneumococci are also capable of severely injuring the blood



ls and of inducing hemorrhages in experimental animals. Since rough pneumococci are readily phagocytosed whereas smooth cultures are not, resistance to phagocytosis appears to be one factor in the virulence of encapsulated organisms. Pneumococci possess to a marked degree the property of invasiveness, and they readily invade the blood stream. Bacteremia in pneumonia patients is associated with a mortality higher than that which occurs in the absence of demonstrable invasion of the blood stream.

**Lobar Pneumonia.** Lobar pneumonia characteristically involves one or more lobes of lung tissue. Following their introduction into the lung via the air passages, the pneumococci become established in an area about the terminal bronchiole, multiply rapidly and within a few hours spread throughout the lung segment. The infected area is extended by passive migration of the pneumococci from one bronchiole to another and alveolus in the edema fluid which accumulates in response to the tissue damage. Phagocytic cells, both polymorphonuclear leukocytes and macrophages, are present in the exudate and, as will be seen, are chiefly responsible for clearing of the infection. In favorable cases antibodies are detectable by the end of the first week, spread of the lesion ceases and the lung begins to be cleared of exudate by a process known as resolution, in which the pneumococci, cellular debris and exudate are in part dissolved and are expectorated in the sputum. The infection may be aborted or resolution hastened by proper treatment.

Pneumonia tends to occur particularly in persons whose general resistance is lowered, as by malnutrition, exposure to cold, concurrent infection and alcoholism, although in many instances no predisposing factors are evident.

**Incidence of *Pneumococcus* Types in Pneumonia.** Numerous surveys have indicated some variation in the frequency of the several types in different series of cases of pneumonia, in different localities and at different times. The variation observed is, however, minor and it is clear that a small number of pneumococcus types is responsible for the majority of illnesses. Recent figures indicate that Types I, II, III, IV, V, VI, VII, VIII, XIV and XIX are the cause of about three-fourths of the pneumococcal pneumonias, all other types being responsible for only the remaining one-fourth of cases. Types I, II and III are particularly frequent and, in addition, produce a more highly fatal infection than do the organisms included in the heterogeneous Group IV.

**Epidemiology of Pneumococcal Infections.** Surveys of the bacterial flora of the upper respiratory tract of normal persons have revealed that pneumococci may be found in nasopharyngeal cultures of as many as 40 to 50 per cent. The frequency of isolations varies considerably, being higher during the cold months of the year, a finding which corresponds to the frequency of pneumococcal infections. The higher pneumococcal types (Group IV) are more numerous among carriers than are Types I, II and III, although all types may be isolated. Many carriers have been found to harbor a single type for relatively long periods of time. The wide distribution of pneumococci provides abundant

sources from which these organisms may be disseminated by droplet infection or by other means.

Pneumonia (both primary and secondary) is a particularly important cause of illness and death among the very young and very old. The prevalence among these groups is roughly five to ten times that in the young adult age groups which are least subject to pneumonia. The disease affects males to a slightly greater extent than females. Both incidence and fatality are higher among the lower socio-economic groups. The annual mortality from all forms of pneumonia in the United States was 51.8 per 100,000 population in 1945, which is about 50 per cent that for 1935 and 1930. Pneumonia ranks among the ten chief causes of death. The disease is world-wide in distribution.

**Antiserum against Pneumococci.** Type-specific antipneumococcus sera are produced by the immunization of horses and rabbits. The antibody-containing globulins from the plasma of such immunized animals are usually purified and concentrated by chemical methods in order to remove extraneous substances and to provide more effective material for therapeutic use. Antipneumococcus serum may be standardized by its ability to protect mice against experimental infection with large doses of pneumococci or by the amount of contained antibody which is precipitable under optimal conditions with the specific SSS.

Antiserum produced in horses, which was made available about the time of World War I, provided the first specific method for treating pneumococcal infections. Later (1937 et seq.), because of certain disadvantages of horse antiserum related to the characteristics of equine antibody, the frequency of sensitivity to horse serum and the difficulty and expense of production, antipneumococcus serum produced in rabbits became widely used. Treatment of pneumonia with specific antiserum has been particularly successful in Type I infections and is effective against many other types, but it has not materially changed the course of infections with Type III.

The mechanisms of immunity in pneumonia have been extensively investigated in both human and animal infections. The function of antibodies appears to be essentially one of intensifying the natural processes of phagocytosis. It has been found that, although phagocytosis occurs in the older central regions of a pneumonic lesion, the peripheral portions in the nonimmune animal contain active pneumococci that spread progressively in the edema fluid. In serum-treated animals, on the other hand, these pneumococci become immobilized by clumping or agglutination, their rate of spread is reduced and phagocytosis is accelerated by opsonization of the cocci. In addition, the SSS is rendered inactive by combination with antibodies.

It should be emphasized that treatment with antiserum is dependent upon identification of the particular pneumococcal type responsible for the disease, a condition which is not always promptly or easily fulfilled. This fact together with the greater efficacy and lower cost of chemotherapy has caused antiserum treatment to be replaced by chemotherapy in the management of pneumonia.

Antipneumococcus serum is administered intravenously in varying amounts



ly 100,000 or more units, after the usual tests for hypersensitivity have been formed. The adequacy of treatment may be indicated by the intracutaneous test for hypersensitivity to SSS (the Francis Test). A positive test, evidenced by immediate formation of an erythematous wheal at the site of injection of the antigen, is obtained in a majority of persons who have an excess of circulating anti-SSS. The test may be positive either in persons receiving adequate treatment with SSS serum or in those who have active immunity against the homologous type of pneumococcus.

**Active Immunization against Pneumococci.** Experimental trials have been made of the vaccination of human beings against Types I and II pneumococci. Vaccination with whole cell vaccines and with preparations of purified pneumococcal saccharides resulted in the production of a positive skin reaction in a high proportion of vaccinated individuals. However, the skin reactions and antibody formation were not directly correlated. Although preliminary trials are suggestive of the preventive value of vaccination against pneumonia, the procedure has not been widely adopted and conclusions as to its value are at present unjustified. In view of the type specificity of immunity, the prevalence of multiple types of pneumococci is a discouraging aspect of vaccination. Active immunization of humans is, of course, well established.

**Chemotherapy.** The pneumococci are sensitive both to the sulfonamide drugs and to penicillin, but are less so to streptomycin. The value of aureomycin is not established. Among the sulfonamide drugs that have been widely used in the treatment of pneumonia, sulfapyridine, sulfathiazole and sulfadiazine appear to be equally effective. However, the lower toxicity of the -thiazole and -diazine derivatives makes them the drugs of choice. The efficiency and low toxicity of penicillin are now well established, and use of this antibiotic is widespread. The efficiency of chemotherapeutic and antibiotic agents against all types of pneumococci, the expense of treatment and the rapidity of recovery are truly remarkable. The development of drug-fast strains of pneumococci has been recorded and presents a potential hazard, against which the availability of several therapeutic agents is a partial safeguard.

Both the sulfonamide drugs and the antibiotics appear to act as bacteriostatic agents which interfere with the multiplication of pneumococci in the infected tissue. Recovery is then accomplished by the mechanisms of phagocytosis, often in the absence of demonstrable specific antibody. Indeed, recent investigations indicate that in the presence of a substrate, such as is provided by the lung surface, phagocytosis of drug-inhibited pneumococci proceeds in the absence of specific antibody.

**Secondary Pneumonia.** Pneumonia secondary to other disease, such as influenza, whooping cough, measles and certain noninfectious diseases, is a serious and often terminal complication. The disease tends to be a bronchial pneumonia involving in a patchy fashion the terminal elements of the bronchial tree and the surrounding lung tissue, and it does not typically spread throughout a single lobe of the lung as does primary lobar pneumonia. The etiological agents of broncho-

pneumonia also differ from those of lobar pneumonia. The pneumococci (chiefly the higher types) are responsible for only a small proportion of cases, and other bacteria, particularly hemolytic and nonhemolytic streptococci, *Hemophilus influenzae* and *Staphylococcus aureus*, are found in a high percentage of cases. The predisposition to infection of the lungs which accompanies such diseases as measles, whooping cough and influenza must be considered in the care of patients in that opportunities for infection from other patients and personnel with secondarily invading organisms are to be avoided. For this reason, isolation in individual rooms and cubicles rather than in wards is preferred. The advent of chemotherapy and treatment with antibiotics has greatly changed the outlook in bronchopneumonia.



## THE GRAM-NEGATIVE COCCI OF MEDICAL IMPORTANCE—THE GENUS NEISSERIA

The importance of the Neisseriae is largely due to the pathogenicity of two members of the group, the meningococcus (*Neisseria meningitidis*) and the gonococcus (*Neisseria gonorrhoeae*). In addition to these pathogenic members, the genus includes several rather fastidious species of gram-negative diplococci of which some are parasitic. The first of the group to be described was *N. gonorrhoeae*, which was observed by Neisser in 1879 in smears of pus from gonorrhea patients. The meningococcus was isolated by Fiechter (1887) from the spinal fluid of patients having meningitis. The non-pathogenic members of the group were first recognized in cultures from the nasopharynx.

**Morphology.** The Neisseriae typically are small flattened cocci arranged in pairs with the flattened or sometimes concave face adjacent to each other, so that they resemble pairs of coffee beans or kidney beans (Fig. 147). Cells grouped in tetrads and clusters are sometimes seen, and in some species the cells may be surrounded by a thin capsule, although capsules are not a constant feature. The Neisseriae stain well with the usual bacterial dyes, are decolorized by the Gram method, and are not acid-fast, do not form spores and are nonmotile.

In general, the members of the group require complex or enriched media for growth, although some of the nonpathogenic members produce colonies on nutrient agar. After 48 to 72 hours' incubation the colonies of the latter organisms are usually 1 to 4 mm. in diameter with slightly irregular margins. They are

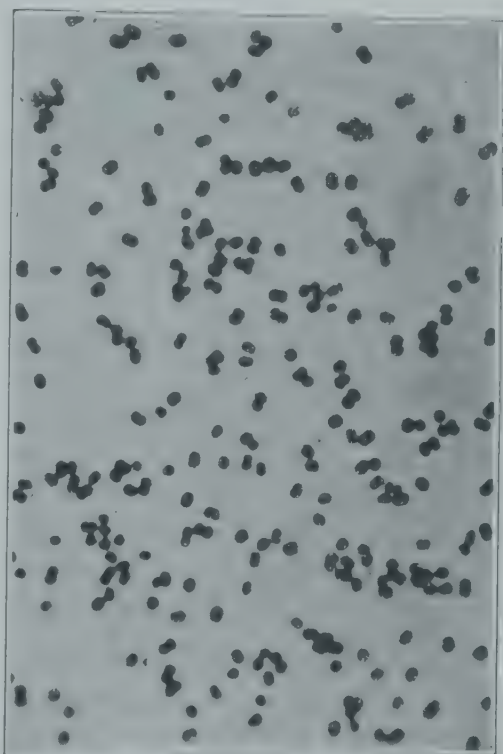


Fig. 147. Smear from culture of *Neisseria gonorrhoeae*. (Magnification approximately  $\times 2,000$ .) (Kral.)

opaque to transmitted light and may be either granular and smooth or rough. The color of the colonies varies from grayish white (*N. catarrhalis* and *pharyngis sicca*) to a bright greenish yellow (*N. flava*). On the other hand, meningococci and gonococci require enriched media for growth. The colonies of these organisms average about 1.0 mm. in diameter and are translucent, smooth, round and convex. Colonies of both the meningococcus and the gonococcus give a positive oxidase test (see below), a reaction of great importance to the isolation of these organisms from mixed culture.

**Physiology and Metabolism.** As a group the Neisseriae are aerobic organisms. However, growth of the meningococcus and the gonococcus is improved by the presence of an increased amount of  $\text{CO}_2$ , so that cultures for the isolation of these organisms are incubated in a sealed jar containing about 10 per cent of this gas.

The temperature range of growth is restricted, particularly of the meningococci and gonococci, which do not develop below  $30^\circ \text{C}$ . Indeed, these organisms do not long survive exposure to temperatures below body temperature, and material for culture should be kept warm during transit from the ward to the laboratory. Some of the nonpathogenic pharyngeal organisms, however, grow readily at room temperature.

The Neisseriae are killed within a few hours by drying and in a few minutes by heating at  $55^\circ$  to  $60^\circ \text{C}$ , or by exposure to the usual disinfectants, such as phenol, mercuric chloride and silver salts. The latter is of particular importance since the silver salts, *e.g.*, silver nitrate, may be used on the body for prevention of infection. Thus in the **Credé treatment**, a 1 per cent solution of silver nitrate is dropped into the eyes of newborn infants to prevent the serious gonorrheal ophthalmia. The solution must be removed within a few minutes to prevent chemical injury. The meningococci and gonococci are particularly autolytic, and that cultures die out easily in the laboratory.

In recent years the medium of choice for isolation of meningococci and gonococci has been an infusion agar containing heated blood (chocolate agar). However, growth may be obtained in casein-hydrolysate media and in media composed of amino acids, glucose and salts. A number of strains of gonococci require glutamine and choline in addition to the nutrient materials. Despite these relatively simple growth requirements, the organisms ordinarily fail to develop on nutrient agar, possibly because of some inhibitory substance in the medium.

The Neisseriae are not very active physiologically. They produce acid from some sugars, but attack few other nutrients. The fact that all members of the group give the **oxidase reaction** is particularly important to their isolation and recognition. In this test, a positive reaction of which depends upon production of an enzyme known as indophenol oxidase, a chocolate agar plate culture of suspected material is flooded with a 1.0 per cent solution of tetramethyl-phenylene-diamine and excess solution is poured off. In a positive test the colonies become purple in color. Recognition of the different species of the genus is based



their fermentation reactions, a summary of which is given in the following

TABLE 11. TYPICAL CULTURAL REACTIONS OF IMPORTANT NEISSERIAE

ORGANISM	COLONY COLOR	GROWTH AT ROOM TEMP.*	FERMENTATION		
			GLUCOSE	MALTOSE	SUCROSE
<i>meningitidis</i>	gray	o	A	A	—
<i>menorrhoeae</i>	gray	o	A	—	—
<i>catarrhalis</i>	gray	+	—	—	—
<i>pharyngis sicca</i>	gray	+	A	A	A
<i>flava</i>	yellow	+ or o	A	A	A or —
<i>luzescens</i>	yellow	?	—	—	—

On nutrient agar.

## THE MENINGOCOCCI AND MENINGITIS

Infection of the meninges, that is, of the membranes covering the brain, may be caused by any of a number of microorganisms, important among which are meningococci, streptococci, pneumococci, staphylococci and the influenza virus. Of these organisms the meningococci, properly referred to as *N. meningitidis* or *N. intracellularis*, are responsible for primary or epidemic cerebrospinal meningitis. This disease, which begins innocently enough with the characteristics of an upper respiratory or gastro-intestinal infection, within a few hours progresses to a severe fever with delirium, stiffness of the neck and back and other signs of invasion of the coverings of the brain. At this time meningococci may be found in abundance in the purulent spinal fluid. Although they are best known as the cause of meningitis, the meningococci at times are found in blood infections (septicemias), and in infection of the joints. Indeed, septicemia and arthritis frequently accompany meningococcal meningitis. A less acute or chronic meningococcal infection sometimes occurs.

The natural habitat of the meningococcus is the nasopharynx of man, and it appears likely that upper respiratory infection is far more frequent than the more serious meningococcal meningitis or septicemia. Cultural surveys have revealed that the carrier state is variable but frequent, at times as many as 10 to 15 per cent of persons having positive cultures. The carrier rate is in general higher during the winter and spring than in the summer.

**Epidemiology of Meningococcal Infection.** Meningitis caused by organisms other than the meningococcus does not occur in epidemic form. The meningococcal disease, on the other hand, has been present in widespread epidemic cycles during the past century. Recent epidemic years include those of World War I (1914-1918), 1928-1930, 1935-1936 and 1943-1944. During

recent epidemics the number of reported cases has been four to nine times number in nonepidemic years. In contrast to the high ratios (up to 50 per cent cases) dying in earlier epidemics the fatality in recent outbreaks has been about 16 per cent, a result undoubtedly due to the introduction of chemotherapy in meningitis.

Meningococcic meningitis occurs in wide areas of the world. In temperate climates the incidence is high during the cold months of the year, often reaching a maximum in the spring, facts which correlate with infection by the respiratory route and transfer of the organisms by droplet infection. The disease tends to affect children more frequently than it does adults. However, epidemics have often occurred in army camps.

**Types of Meningococci.** By immunological methods, particularly agglutination and the agglutinin-absorption reactions, using specific sera, several types of meningococci have been recognized. At present the schema of Gordon and Murray, which recognizes four types (Types I, II, III and IV), is the most widely accepted. Numerous cross-reactions occur, so that Types I and II, which are closely related, are commonly placed together in a large Group I, and Types II and IV, also related, are classed in a more heterogeneous Group II. Epidemiologically, Group I strains are associated with severe epidemic meningitis, whereas those strains in Group II are more often found in sporadic, less severe infections and in carriers. In addition to their specific antigens, the meningococci have a broad group antigenic relationship with the gonococci, but not with other members of the Neisseriae.

**Virulence of Meningococci.** The virulence of meningococci is not easily demonstrated in the laboratory. Strains rapidly lose virulence during cultivation on artificial media and laboratory animals are refractory unless excessively large doses are injected or the organisms are inoculated in a suspension of mucin. Using the latter method fatal infections may, however, be produced in mice with a very small number of virulent organisms. There is considerable variation in the virulence of freshly isolated strains of meningococci, the number of organisms of one culture required to kill mice often being many times that of another culture. By means of the lyophil (the rapid drying of cultures from the frozen state) and the mucin techniques virulence may now be preserved for long periods of time.

The virulent strains of meningococci are smooth, and the organisms usually produce capsules. Such strains appear to be more resistant to phagocytosis than do the rough avirulent cultures.

**Meningococcal Toxin.** Meningococcal cells and their autolysates in large doses are toxic for a number of laboratory animals. The effect of such preparations is, however, irregular. In addition, the filtrates of young cultures (24 to 48 hours old) of some strains have been found to contain a toxic material which produces a severe meningeal inflammation after cisternal injection into animals and which gives rise to a skin reaction in man. This "exotoxin" is antigenic, and antitoxin against it has been used in the treatment of patients having meningitis.



ever, not all strains are toxigenic and in positive cultures the toxin is present during the first few days of growth. The question of whether this material presents a true exotoxin or not is problematical.

**Meningococcal Antiserum.** Both rabbits and horses may be immunized successfully against meningococci. The serums for clinical use are usually polyvalent in that they are prepared in horses against several strains representing different groups and types. Serums for treatment of patients are purified and concentrated by chemical methods. The therapeutic value of the serum is determined by the mouse protection test in which the protective value of unknown serum against many fatal doses of meningococci is compared to that of a known antiserum. Prior to introduction of chemotherapy, treatment with antiserum was the standard method of specific treatment of meningococcal infection. The fatality in a recent series of patients treated with antiserum was approximately 25 to 50 per cent, a marked improvement compared to the almost universal fatality of untreated meningitis. Serum should be given early during the disease in order to obtain maximal benefits.

**Chemotherapy.** Meningococci are susceptible to the sulfonamide drugs, particularly to sulfapyridine, sulfathiazole and sulfadiazine. Since the advent of chemotherapy the fatality from meningitis has been reduced to from 3 to 15 per cent. The sulfonamide drugs have, therefore, practically replaced serum treatment, although combined drug and serum therapy is sometimes used.

Also, the meningococci are among the organisms naturally highly susceptible to penicillin, which appears to be about of equal value with the sulfonamide drugs in the treatment of meningitis. Meningococci are also naturally susceptible to streptomycin, which may be used in the treatment of infections.

It must be remembered that, following exposure to the sulfonamides and to penicillin and streptomycin, the organisms may become resistant to many times the concentrations that were originally inhibitory. This fact is important in the therapy of disease with these agents, and makes it particularly desirable to have available more than one agent effective against the bacteria.

## THE GONOCOCCI AND GONORRHEA

The gonococci are strictly human parasites. Although they were first described in the purulent lesions of gonorrhea and are best known as the causative agent of that disease, they at times invade mucous membranes other than those of the genito-urinary tract and are sometimes found in infections of the deeper tissues, such as purulent arthritis and meningitis. In the adult, gonococcal infection is typically a venereal disease transmitted by sexual contact (see Chapter 10). In the male the organisms first invade the urethra, producing an acute purulent infection which, without proper treatment, progressively extends through the urethra to the seminal vesicles and the tubules of the epididymis. In the female, an acute purulent inflammation of the genito-urinary tract also occurs.

the infection typically extending from the vagina and uterine cervix to fallopian tubules, or oviducts, and the surrounding tissues.

The acute disease is in most instances of relatively short duration, but usually it is followed by a prolonged chronic infection characterized by local tissue destruction, urethral stricture in the male and abscess formation in the female. In some instances infection is spread to other tissues of the body. Both the male and female gonorrheal patient without proper treatment may be infectious for long periods of time after subsidence of the acute disease. Although immunized carriers in the strict meaning of the word are unknown in gonorrhea, chronically infected individuals play a similar epidemiological role. Infection in the female assumes particular importance since the organisms may be transmitted congenitally from mother to child during delivery of the baby, in whom gonorrheal ophthalmia or *ophthalmia neonatorum* is produced. Gonorrheal infection of the eyes is, indeed, one of the important causes of blindness. Gonococcal infection may be transmitted by personal contact other than venereal and fomites, such as towels, clothing, bedding and lavatories. The gonorrheal patient may by uncleanly habits transfer infection to his own eyes or to other persons. Furthermore, gonorrheal infection of the lower genital tract (vulvo-vaginitis) sometimes occurs in young girls as the result of transmission by lavatory towels, and personal contact. This infection may reach epidemic proportions.

**Pathogenicity.** The mechanisms of disease production by gonococci are obscure. These organisms are nonpathogenic for lower animals, so that laboratory investigations are limited. They do not produce exotoxin, although the substance is pyogenic and in large quantities is fatal to laboratory animals.

**Laboratory Diagnosis of Gonococcal Infection.** The bacteriological diagnosis of gonorrheal infection is made by the demonstration of the organisms by microscopic and cultural methods. In smears of gonorrheal pus stained by Gram's method (Fig. 140), the organisms typically are seen within leucocytes. The smear diagnosis may be made with greater certainty in the male than in the female and is most often positive in the acute infection. The methods used for the cultivation and identification of gonococci have already been discussed. It is important to emphasize the necessity for the proper handling of material for culture and to point out that cultures frequently are positive when direct smears fail to demonstrate the organisms.

Many attempts have been made to develop serological methods for the diagnosis of gonorrheal infection. Of these, the complement-fixation reaction using the patient's serum, has been of some value, particularly in the diagnosis of infections of the deeper tissues, such as arthritis. However, the complement-fixing antibodies are not uniformly present, and the test has not replaced bacteriological diagnosis.

**Types of Gonococci.** Although different types of gonococci have been recognized in recent years by several investigators, the group is heterogeneous, and immunological classification is not firmly established and has not assumed practical importance. On the basis of colonial characteristics and the absorption



inins. Types I and II may be differentiated. In addition to their specific  
ns, the gonococci are related immunologically to the meningococci.

**treatment of Gonococcal Infection.** Serum effective against these or-  
ns has not been developed. Consequently until recent years treatment and  
tion consisted largely in the local application of inorganic and organic  
compounds and in nonspecific measures. Fortunately the gonococci, like  
meningococci, are sensitive to the sulfonamide drugs and to penicillin and  
omycin. Treatment with these agents will in most instances eliminate the  
ion within a few days and has reduced the danger of chronic infection and  
lications. However, some infections are less easily eradicated, and the  
occi may acquire resistance to these therapeutic agents.

## THE SPORE-FORMING BACTERIA— THE BACILLACEAE

The bacteria included in the family *Bacillaceae* are rod-shaped organisms remarkable for the production of highly resistant spores. Many species are saprophytes which are widely distributed in the soil and water, whereas others are important pathogens, still others are used in the industrial production of alcohols and related chemical substances and some are responsible for spoilage of foods. The bacilli were early associated with disease in man and animals, members of the group having been found responsible for anthrax, tetanus or lockjaw, gas gangrene and botulism, following Koch's conclusive demonstration of the role of bacteria in disease when he succeeded in producing anthrax in animals by inoculation of a pure culture of the bacilli, and Pasteur's studies of vaccination against anthrax. Soon thereafter the causative agents of tetanus (1884), gas gangrene (1877) and botulism (1896) were found to be spore-forming organisms.

**Morphology.** The spore-forming rods are among the larger bacteria, individual cells measuring from 0.3 to 3.0  $\mu$  in width and up to 9  $\mu$  in length. The cells are elongated with either blunt or rounded ends and are often arranged in chains. The majority of species possess flagella and hence are motile (a notable exception being *Bacillus anthracis*). The cells of some species are encapsulated. The distinguishing characteristic of the group is, however, the formation of highly resistant endospores. These spores are smaller in size than the vegetative cells and may be either spherical or slightly elongated in shape. They may be located centrally, eccentrically or terminally along the long axis of the cell. Although vegetative cells are not acid-fast and stain well with the dyes usually used for staining bacteria, the spores are mildly acid-fast and are not colored by ordinary dyes. The vegetative cells are gram-positive in young cultures. The color and morphology is distinctive for the species.

**Resistance.** The vegetative cells of the Bacillaceae are killed by heating 55°–60° C for one hour or at boiling temperatures within a few minutes, and are destroyed by common disinfectants in a short period of time. The spores, on the other hand, are able to withstand boiling for some minutes, occasionally for several hours, and are relatively unaffected by the usual disinfectants. They are, however, killed by strong alkali, such as 10 per cent sodium hydroxide or lye, and, in aqueous solutions, within a few minutes by steam under pressure at 120° C.



or similar conditions, somewhat longer periods of heating are required to destroy spores in infected tissues or in heavy materials, such as foods, through which they penetrate slowly. The resistance of the spores of different species is variable, that of *Clostridium botulinum* being particularly great, whereas the spores of *B. anthracis* are less resistant to both heat and disinfectants. The sporulating bacilli are customarily classified into two genera: the genus *Bacillus* including those species which grow aerobically, and the genus *Clostridium*, which includes the anaerobic species.

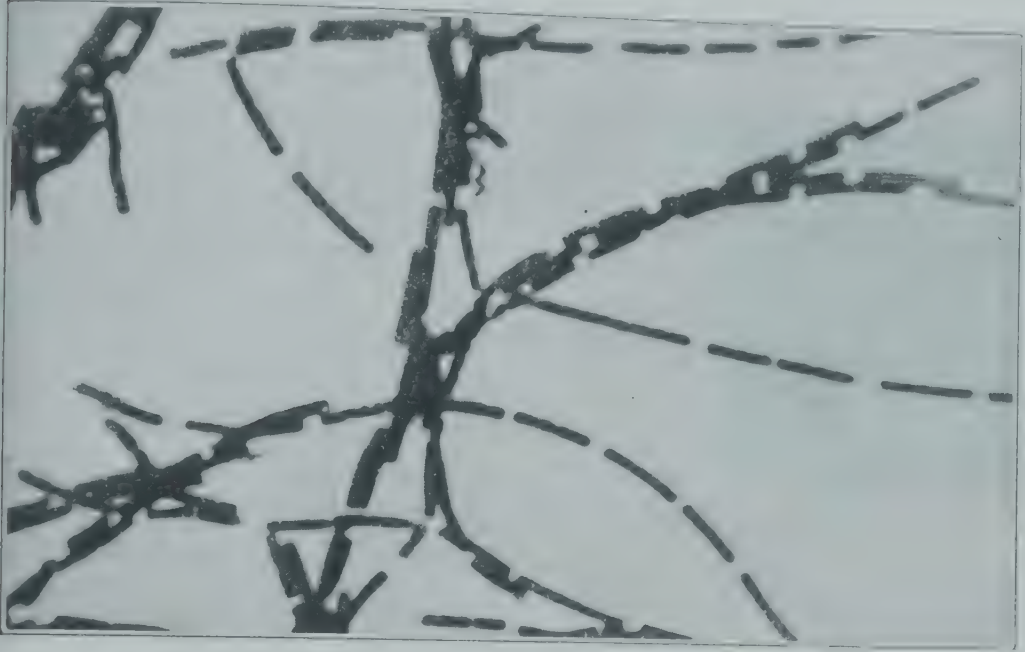


Fig. 148. *Bacillus mycoides*. Photomicrographs at exceptionally high magnification showing chains of bacilli. (Magnification approximately  $\times 2,500$ .) (Kral.)

## THE GENUS BACILLUS

The genus *Bacillus* includes a number of saprophytic, mesophilic and thermophilic bacilli and the one pathogenic species, *Bacillus anthracis*.

**The Nonpathogenic Bacilli.** Although many species of aerobic saprophytic bacilli have been described, not all species are clearly differentiated. These organisms generally grow well on ordinary nutrient media at room ( $20^{\circ}$ - $22^{\circ}$  C) or body ( $37^{\circ}$  C) temperatures, and some thermophiles grow at temperatures above  $50^{\circ}$  C. They ferment sugars with production of acid and are generally proteolytic. Most species are motile. A few varieties produce pigmented, usually brownish growth. The group is of medical importance because of the wide distribution of its members and hence the frequency of their presence as contaminants, and because they must be distinguished from *B. anthracis*.

One of the most frequently encountered saprophytic organisms is *Bacillus subtilis* (the hay bacillus), which is widely distributed in dust, soil, water and air. *Bacillus subtilis* is a motile, gram-positive, slender rod with rounded ends. The cells are usually arranged in short chains. The colonies are grayish white

with irregular margins and a rough surface (Fig. 149). *Bacillus mycoides* and *B. megatherium* are also commonly encountered soil and water bacteria. The former produces typically spreading, rhizoid colonies which have a lacy appearance. The latter organism is a large spore-forming rod, the colonies of which are granular with entire margins. Both species are motile. *Bacillus mesentericus* is of practical importance as the causative organism of rosy bread.

**Bacillus Anthracis and Anthrax.** Anthrax is an important, highly fatal disease of lower animals, particularly of the domestic herbivorous cattle, horses, swine, goats and sheep, and secondarily of man. Anthrax is present in almost all countries of the world. Animals usually are infected by ingesting contaminated



Fig. 140. Colonies of *Bacillus subtilis* on blood agar. The granular, irregular colonies are surrounded by a zone of hemolysis ( $\times 2$ ).

food, the bacilli gaining entrance either through the intestinal tract or at the site of small abrasions on the mouth and pharynx. The disease is a septicemia; the blood, tissues and body discharges contain large numbers of the vegetative cells, although sometimes abscesses and carbuncles are formed locally. The organisms sporulate upon leaving the body, and the spores which form they remain viable indefinitely. Pasture may thus prove infectious for many years following the occurrence of anthrax. Furthermore, the organisms may be spread to previously uninfected animals in imported contaminated animal food.

Human infection is almost invariably occupational. Farmers, shepherds, woolsorters, tannery employees and veterinarians become infected from diseased animals and wool, bristles and hides contaminated with spores. Occasionally the use of improperly sterilized articles, such as brushes, made from contaminated materials may result in the disease, and rarely intestinal anthrax occurs following ingestion of contaminated animal products.

Human anthrax may be divided into two main types, the **cutaneous** (malignant pustule) and the **pulmonary** (wool-sorter's disease). In the cutaneous type of infection, which is the more common variety, invasion occurs through abrasions following contamination of the skin with anthrax bacilli or spores, so that the exposed areas of skin, particularly the face and hands, are the sites of the lesions. The cutaneous lesion begins as a red macule, which progresses within a few days to a hemorrhagic vesicle or blister, then to a firm indurated lesion with a black crust or eschar. The lesion lasts several weeks. In the pulmonary type a pneumonia is present which must be differentiated from other acute infections of the lungs. Pulmonary anthrax, which is almost exclusively a disease of workers in the woolen industry engaged in handling contaminated wool, is acquired by inhalation of spores. In fatal infections of both types, septicemia and widespread invasion of the tissues are present.

The laboratory diagnosis of anthrax is made by the demonstration of typical



isms by smears, cultures and animal inoculation tests with material from lesions, exudates and blood. The organisms are usually abundant in infected tissues and may be readily demonstrated by the above methods. Antigens of anthrax bacillus may be demonstrated in infected hides and tissues by the **li thermo-precipitin test** with specific immune serum in which boiling extracts of the material being examined are used as antigen. Precautions should be rigorously observed in handling suspected material in order to avoid contamination of the soil by animals dead of anthrax and to prevent infection of personnel engaged in the examination.

**Bacillus Anthracis.** In the blood and tissues of infected animals *Bacillus anthracis* appears as a blunt-ended, gram-positive bacillus, arranged singly, in pairs or in short chains. Spores are not formed in the body, but they are produced in tissues after death of the animal and in cultures. The cells of virulent strains are nonencapsulated and are nonmotile. Colonies of such cultures on agar are 2-3 mm. in diameter, have irregular margins and are rough. Avirulent cultures, conversely, do not possess capsules and the colonies are smooth to mucoid. Rough to smooth transition (virulent to avirulent) occurs readily in cultures grown for prolonged periods at high temperatures, a fact which is, indeed, used for production of attenuated vaccines for veterinary use. Virulence may be restored to such cultures by animal passage. Although most strains produce centrally located spores, mutants may be developed which have lost the ability to sporulate. Asporogenous mutants appear to possess the virulence of the parent culture.

*Bacillus anthracis* ferments glucose, maltose and sucrose readily and salicin slowly, and it is slowly proteolytic. The cultural characteristics do not suffice, however, to distinguish *B. anthracis* from nonpathogenic bacilli. The growth requirements are simple in that cultures will develop on amino-acid media without presence of added vitamins.

*Bacillus anthracis* is pathogenic for man, the domestic herbivores and for wild game, pigs, mice and rabbits. In the latter animals the infection is usually fatal in a few hours to two days following inoculation, and is characterized by a hemorrhagic lesion and a septicemia. The organisms are usually present in numbers sufficient to suggest death by an overpowering septicemia. However, in some cases few organisms are observed and death must be attributed to other, as yet obscure, factors. Toxins have not been demonstrated.

In contrast to the capsules of most bacteria, which are composed of carbohydrate material, those of the anthrax bacillus are a polypeptide of the amino acid  $\gamma$ -glutamic acid, and hence are protein in nature. Both the capsular and somatic (somatic) antigens of the anthrax bacillus react specifically with anti-anthrax serum, although some cross-reactions may be obtained with the somatic antigens of other bacilli.

**IMMUNITY.** The mechanism of the natural immunity against anthrax, which is present particularly in rats, dogs and fowl, has occasioned a great deal of speculation. It was early observed (Pasteur) that the resistance of fowl could be increased by reducing the body temperature, a fact probably correlated with de-

creased destruction of organisms by phagocytosis. Bactericidal activity may be present in the blood, as in the case of the rat and the rabbit, although bactericidal activity is not directly correlated with resistance to infection, as is shown by the susceptibility of the rabbit.

Acquired immunity may be produced by vaccination. The Pasteur method consisted of two inoculations, the first of organisms attenuated by growth at 42.5° C for 15 to 20 days, the second of a less attenuated culture similarly treated for 10 to 12 days. Other vaccines composed of heat-attenuated organisms or spores combined with immune serum have been found to be effective and relatively safe. Vaccines composed of killed organisms are apparently ineffective.

**TREATMENT.** Immune serum prepared in animals was until recently the standard method for specific treatment of anthrax in man, and was employed together with the arsenical drugs and sometimes local excision or cautery in treatment of human disease. Recently, treatment with penicillin alone or combined with sulfonamide drugs has proved efficacious experimentally and clinically, and in all likelihood will replace older methods of treatment.

**PREVENTIVE MEASURES.** Preventive methods have consisted in regulations concerning disposal of infected carcasses, shipment of hides and wool from infected areas and the heat sterilization of brushes. Chemical disinfection is largely ineffective against spores and has largely been discontinued in favor of heat sterilization of animal products where this is possible and by the use of closed processes and exhaust ventilation in industry.

## THE GENUS CLOSTRIDIUM

The clostridia are widely distributed saprophytes of soil and water and are parasites of man and animals. A number of species produce highly potent toxins and are important pathogens. Others play an important role in nitrogen fixation and proteolysis in nature and some species are used in industrial fermentation. All members of the genus are anaerobic, although *Cl. histolyticum* may develop slightly in the presence of air.

The saprophytic species, including *Cl. botulinum*, may be cultured from soil samples taken from widely separated geographical regions. The members of the gas gangrene group and *Cl. tetani* commonly are isolated from the intestinal contents and feces of man and animals and are found in abundance in human and manured soils, but are infrequently found in unpolluted or virgin soils. Their natural habitat is in all likelihood the intestinal tract, although some bacteriologists consider them to be natural residents of the soil.

**Metabolism.** The outstanding metabolic characteristic of the clostridia is their inability to develop in the presence of oxygen. They grow readily, however, in media reduced by the addition of chemical substances or by exclusion of oxygen from the atmosphere (see Chapter 11 for a discussion of anaerobiosis).

Many species have been grown in chemically defined media and with a few exceptions they require added growth factors or vitamins. Thus *Cl. sporogenes*



of the common proteolytic species, needs biotin to replace an ill-defined "sporogenes vitamin." *Clostridium perfringens*, an important cause of gas gangrene, requires pantothenic and pimelic acids for growth, and riboflavin and nicotinic acid for toxin production. Strains of *Cl. botulinum* vary greatly in vitamin requirements, some being similar to *Cl. sporogenes*, others presumably requiring no added vitamins. The growth requirements of *Cl. tetani* are particularly complex and other clostridia have been found to need thiamine, para-aminobenzoic acid and asparagine, although pantothenic acid and *p*-aminobenzoic acid have been necessary in most instances. One or more of the above acids are required as sources of nitrogen by the majority of species.

A number of clostridia are of agricultural and industrial importance. For example, *Cl. pasteurianum* is able to fix atmospheric nitrogen and *Cl. acetobutylicum* and *Cl. butylicum* are used in the commercial production of butyl alcohol and acetone from fermentable carbohydrate. The products of fermentation in general are volatile acids with abundant gas formation. Thus the fermentation of lactose in milk by *Cl. perfringens* (*Cl. welchii*) is characterized by disruption of the acid curd by gas, giving rise to the typical "stormy" fermentation.

Many clostridia are actively proteolytic and produce indole, liquefy gelatin and attack many natural and denatured proteins and amino acids.

In at least one instance the enzyme system of the bacterium has been shown

to be closely related to toxicity; the lecithinase of *Cl. perfringens* has been found to be identical with one of the toxic fractions of this organism. The ability of this and a few other species to attack lipoproteins, giving rise to water-insoluble emulsoid material, is the basis of the formation of an opalescence in human serum on addition of toxic filtrates (Nagler reaction). A more well defined opacity is produced in lecithovitellin extracted from egg yolk and this may be observed as a halo about colonies on egg yolk agar. The lecithovitellin or Nagler reaction is not specific for *Cl. perfringens*, since it is given by several species of the gas gangrene and botulinum groups.

**Pathogenicity.** The clostridia which are able to cause infection in the human and animal body, e.g., those causing gas gangrene and tetanus or lockjaw, do not invade through the intact skin or mucous membranes. In the event that



Fig. 150. Colonies of *Clostridium sporogenes* grown on nutrient agar for four days under anaerobic conditions. (Courtesy of Dr. G. M. Dack.)

they are introduced into wounds, however, they may give rise to severe infection and fatal general toxemia. Infection is most likely in wounds contaminated with the soil or those in which maceration and death of soft tissue has occurred. War wounds and compound fractures have thus been particularly subject to the development of gas gangrene and tetanus. The organisms of tetanus and gas gangrene produce exotoxins to which their pathogenicity is attributed entirely or in part. *Clostridium botulinum*, which is responsible for the highly fatal food poisoning botulism, is doubtfully classed as pathogenic; the disease is strictly a toxemia resulting from ingestion of preformed toxin. The organism itself is a saprophytic soil organism which does not invade the body.

### GAS GANGRENE

Gas gangrene is an infection, which may be highly fatal, associated with war wounds and to a lesser extent with civilian injuries. The disease is characterized by extensive local death and destruction of tissue with purplish red discoloration, edema, a foul odor and, as the name implies, the formation of gas within the lesion. Growth of the causative organisms is favored by macerated tissue or débris within the wound. Generalized toxemia is usually present; septicemia is not rare. Infections similar to those encountered in war wounds may complicate surgical wounds, appendicitis with peritonitis and the like. In general such infections may occur in those conditions in which injured tissues are subject to fecal or soil contamination.

**Bacteriology.** The first descriptions of a *Clostridium* later incriminated in human gas gangrene were made by Pasteur in 1877 when he isolated the "*Vibrion septique*" from the blood of a calf, and by Koch and Gaffky, who in 1881 separately described a similar organism from "malignant edema" of animals. Thereafter several species of clostridia were isolated either in pure or mixed culture from gangrenous lesions of man. Thus in 1891 Achalme, in 1892 Fraenkel and Nuttall and in 1893 Fraenkel described the organism now known as *Clostridium perfringens* (*Clostridium welchii*), and Novy in 1894 isolated *Clostridium oedematiens*. By 1914 the importance of clostridia in the etiology of gas gangrene was well recognized, although the separation of species was incomplete. Other organisms, such as *Esch. coli* and *Proteus sp.*, were found to produce similar infections. During World War I, gas gangrene of war wounds constituted one of the most important medical problems and was intensively studied by Weinberg and Séguin and others. The results of these and other studies are briefly summarized as follows:

Gas gangrene usually results from clostridial infection, either by the organism in pure culture or, more often, in mixture with other aerobic or anaerobic bacteria. The clostridia most frequently involved are *Cl. perfringens* (*Cl. welchii*), *Cl. septicum* (*Vibrion septique*), *Cl. oedematiens*, *Cl. sporogenes*, *Cl. fallax*, *Cl. histolyticum*, *Clostridium aerofœtidum* and *Cl. sordellii* (*Cl. bifementans*). These organisms are active fermenters and many are toxigenic.

A small proportion of gangrenous wound infections (possibly 10-20 per cent)



by organisms other than the clostridia. In both the clostridial and nonclostridial gas bacterial synergism, *i.e.*, the additive effect of mixed bacterial cultures, seems an important factor, although a number of the clostridia in pure culture are capable of producing extensive gangrenous tissue destruction.

**Clostridium Perfringens** (*Clostridium welchii*, *Fruenkel's Bacillus*), *Clostridium perfringens* is a nonmotile, relatively large, gram-positive rod which occurs singly and in pairs and chains. Central spores are formed in neutral and reducing media, but not usually in the animal body or in media containing fermentable carbohydrate. Capsules are observed about organisms in smears from abscesses.

Colonies on the surface of anaerobic blood agar plates are 2 to 5 mm. in diameter, white, smooth, entire and usually surrounded by a zone of clear hemolysis. Deep colonies are biconvex and not distinctive. On egg yolk agar a positive lecithovitellin reaction is given. The organism is actively fermentative, producing abundant acid and gas from dextrose, lactose, maltose and sucrose and sometimes from inulin and glycerol. In milk, typical "stormy" fermentation is produced without digestion of the clot.

**Toxicogenicity.** Cultures of *Cl. perfringens* isolated from gas gangrene and the intestinal tract of man have been found to be related immunologically and antigenically with cultures isolated from lamb dysentery, struck of sheep and botulism of sheep. These related organisms are often referred to as *Cl. welchii*, Types A, B, C and D respectively. Careful investigations have revealed that these bacteria produce one or more of seven toxins which are immunologically specific. The sharing of these toxins is responsible for the interrelationships between the toxigenic types. The human pathogen (*Cl. perfringens* or *Cl. welchii* type A) produces three toxic components: the  $\alpha$  toxin which is hemolytic, irritating to the skin of rabbits and guinea pigs and lethal to mice; the  $\epsilon$  toxin, a thermolabile and oxygen labile, hemolytic and lethal toxin and  $\eta$  toxin which is lethal only. The  $\alpha$  toxin has been shown to be a lecithinase.

The toxin of *Cl. perfringens* is of relatively low potency in comparison to that produced by *Cl. botulinum*, and it is immunologically specific. Antitoxin produced in horses against filtrates of cultures has proved of great value in the treatment and prevention of gas gangrene in man. In addition to the toxins described above, *Cl. perfringens* produces abundant hyaluronidase.

Classification of *Cl. welchii* into immunological subtypes by means of the agglutination reaction and into fermentative types has been proposed but is not of practical value at the present time.

**Pathogenicity.** *Clostridium perfringens* is the most common and important causative agent of gas gangrene of wounds and, as indicated above, it is at times antigenically related to other gangrenous infections and to puerperal fever. In the laboratory, gangrenous lesions may be produced by intramuscular or subcutaneous inoculation of guinea pigs, and both cultures and toxin are highly pathogenic to mice as well as to guinea pigs. Natural infections in domestic animals occur with *Cl. welchii* types B, C, and D.

It must be remembered that *Cl. perfringens*, as well as the other clostridia, does not invade the intact tissues but, rather, acts as an opportunist in which where physical injury or damage by other organisms provides suitable conditions for growth. The presence of *Cl. perfringens* in the intestinal tract and in the soil provides adequate opportunity for contamination of wounds and indicates the necessity for preventive and aseptic precautions.

**Clostridium Septicum** (*Vibrion septicum*). *Clostridium septicum* is a gram-positive, motile, sporulating, strictly anaerobic rod, the cells of which have somewhat pointed ends. Capsules are not formed. Spores are located subterminally or centrally and are formed readily in culture media free of fermentable carbohydrate and rarely in the animal body. The cells are arranged typically in chains within the body but occur singly or in short chains and groups in culture. Colonies have arborescent or rhizoid margins with deep opaque centers.

*Clostridium septicum* ferments carbohydrates with production of abundant gas, is moderately proteolytic in that it produces  $H_2S$  and liquefies gelatin but does not produce indole or digest coagulated proteins.

Cultures may be divided into immunological groups on the basis of cell surface and flagellar agglutinogens. Cross-reactions occur with *Cl. chauvoei*, an anaerobic pathogen, the cause of black leg in cattle and horses.

**Pathogenicity.** *Clostridium septicum* has been recovered not only from human gas gangrene but also from gangrenous and highly fatal infections in domestic animals. In laboratory animals, subcutaneous inoculation is followed by development of an edematous, destructive local lesion and by septicemia which is usually rapidly fatal.

Pathogenicity is related to production of specific toxin, which in relatively large doses is highly lethal. Locally the toxin produces a marked edema and necrosis. Specific neutralizing antitoxin which has therapeutic value has been produced.

**Clostridium Histolyticum.** *Clostridium histolyticum* is of great interest because of its unique pathogenicity. This organism, originally described from war wounds by Weinberg and Séguin, is an anaerobic or microaerophilic, sporulating, gram-positive rod. It grows singly or in short chains, is motile and readily produces subterminal spores in all media. Colonies are granular and rhizoid.

*Clostridium histolyticum* is not saccharolytic. It is strongly proteolytic, rapidly attacking coagulated proteins, casein, gelatin and, of great importance, living animal tissues.

**Pathogenicity.** Intramuscular inoculation of culture or culture filtrate in experimental animals results in extensive destruction and liquefaction of soft tissues. Remarkably, there is little or no evidence of generalized toxemia. In the guinea pig, for example, a rapidly extending local lesion of the lower extremity is usually unaccompanied by general illness until the lesion has spread sufficiently to cause erosion of the abdominal wall. Death usually results from secondary peritonitis. Subcutaneous inoculation leads to a similar, but less severe, lesion and intravenous inoculation is relatively harmless. The mar-



lytic activity of cultures and filtrates seems to be responsible for the pathogenicity of *Cl. histolyticum*, although filtrates are also hemolytic. Large amounts of culture (about 1.0 ml.) and culture filtrate (several ml.) are required to produce typical effects. The active material is antigenic and, although important in comparison to typical exotoxins, is in one sense a toxin.

In natural infections *Cl. histolyticum* is usually found in association with other organisms. It is less often isolated from lesions of gas gangrene than are all other members of the group.

**Clostridium Oedematiens** (*Clostridium novyi*). *Clostridium oedematiens* was originally described from a synergistic infection in the guinea pig, but it has since been recovered frequently from human gas gangrene and has been isolated from infections of domestic animals. In laboratory animals, virulent strains are lethal following intravenous inoculation and give rise to edematous and hemorrhagic lesions after intramuscular or subcutaneous inoculation. Bacteria-free filtrates produce similar effects. Toxic filtrates induce formation of specific antitoxins in horses and rabbits, and this has some value in prevention and treatment of infections.

*Clostridium oedematiens* is a relatively large rod which usually occurs singly or in short chains. Subterminal spores are formed readily in all media and may be found in the tissues. Surface colonies have irregular margins. The organism is mildly saccharolytic and proteolytic.

**Clostridium Sporogenes.** Although *Cl. sporogenes* has been recovered frequently from mixed wound infections and probably contributes to the putrefactive character of such wounds, its pathogenicity is doubtful. It is widely distributed in nature and hence gains ready access to wounds. Experimentally, cultures are relatively avirulent and nontoxic, large doses being required to produce typical effects in animals.

*Clostridium sporogenes* is an actively motile rod with rounded ends, measuring on the average, 0.7 by 3 to 7  $\mu$ . Spores are located subterminally, are oval in shape and bulge the cell. Cells are arranged singly, in pairs or in short chains. Colonies on the surface of solid media are flat with irregular to rhizoid margins and are somewhat "fuzzy" in appearance in deep cultures. The organism is relatively proteolytic and is able to attack some carbohydrates. Coagulated proteins, gelatin and casein are rapidly digested and  $H_2S$  is produced in large amounts.

**Clostridium Sordellii.** *Clostridium sordellii* is a toxigenic anaerobe which has been isolated from a number of wound infections since its description in 1924. The organism is remarkably similar to, if not identical with, *Cl. bifermittans*, a nonpathogenic organism frequently isolated from wounds. *Clostridium sordellii* reportedly is not widespread in nature, although *Cl. bifermittans* has been described from widely different sources. The organism is strongly hemolytic and is in many ways similar to *Cl. sporogenes*.

Filtrates of cultures of *Cl. sordellii* are toxic to animals after intravenous

inoculation. Antitoxin which protects specifically against the effects of filtrates has been prepared.

**Prevention and Treatment of Gas Gangrene.** In addition to immune serums or antitoxins available as mixed gas gangrene antitoxins, sulfonamide drugs and particularly penicillin have been found effective in clostridial wound infection. Although these agents are not equally active against all clostridia their use has been most beneficial.

In general, débridement, *i.e.*, removal of dead tissue and debris which creates suitable conditions for growth of anaerobes, is important in treatment and prevention of these infections. Thorough cleansing, including scrubbing of the entire surgical apparatus, is necessary after treatment of a gangrenous infection. Prolonged heat sterilization of instruments, utensils and linens is required. The presence of the clostridia on raw surgical ligatures made of gut has necessitated rigorous sterilization of these materials during production and regulations of their bacteriological quality in order to prevent infection of surgical wounds.

**Nonclostridial Gangrene.** Gangrenous infections particularly of the skin and underlying tissue and of surgical wounds are at times caused by organisms other than the clostridia. Such infections are encountered in civilian as well as in military wounds. The causative organisms are present in mixed cultures and the spreading gangrenous nature of the lesion appears to result from the synergistic activity of the combined bacteria. Synergistic gangrene has been associated particularly with infection by microaerophilic or anaerobic streptococci and staphylococci (Meleney's gangrene), although associations of mixed anaerobes with members of the enteric group of bacteria, diphtheroid rods and non-hemolytic streptococci have been reported. Fusospirochetal gangrene of the skin follows bites and similar injuries. Impetiginous gangrene appears to result from infection with mixtures of hemolytic streptococci and staphylococci. *Endamoeba histolytica* and mixed bacteria are found in amoebic gangrene.

Despite the use of chemo- and antibiotic therapies, treatment of these infections is difficult and not entirely satisfactory. The infections tend to be prolonged and may be recurrent.

### CLOSTRIDIUM BOTULINUM AND BOTULISM

The disease known as botulism (from L., *botulus*, meaning sausage) has long been recognized as a poisoning resulting from eating spoiled and toxic food. It was not until 1895, however, that van Ermengem isolated the causative organism, *Clostridium botulinum*, and proved the role of it and its toxin in disease production.

The serious symptoms of botulism are entirely attributable to action of the toxin on the motor nervous system, although inconsequential gastro-intestinal disturbances may be present. The characteristic symptoms are thus due to impairment of muscular activity, and include general weakness, double



(dysphagia), difficulty in swallowing, talking, and breathing, and loss of muscular control. Death occurs in from 20 to 100 per cent (usually 60 to 70 per cent) of cases and with few exceptions is due to respiratory failure. There is a remarkable absence of sensory disturbances, and consciousness is usually maintained. The onset may be as early as a few hours or as long as a week following ingestion of toxic food.

**Epidemiology.** The epidemiology of botulism is discussed in detail in the chapter on food poisoning. It is none the less important to point out here that the disease is world-wide in distribution.

As would be expected from the isolation of *Cl. botulinum* from virgin soils collected in widespread geographical areas. The frequency and fatality of outbreaks is, however, highly variable.

Botulism results from eating foods containing preformed toxin and is encountered where preserved subsequently uncooked foods are actually eaten. Thus in Europe preserved sausages and fish and in the United States home-canned vegetables and fruits have been most frequently involved in outbreaks. Epidemics tend to involve a small number of persons, often in a single family, and usually they can be traced to one item of food.

**Clostridium Botulinum.** *Cl. botulinum* is a large rod, 4–6  $\mu$  long and approximately 1  $\mu$  in diameter. The rods are motile by means of peritrichous flagella and produce large subterminal spores which are highly resistant to heat. The colonies tend to

be large, are smooth and glistening and are hemolytic on blood agar.

Cultures grow well on ordinary nutrient media under anaerobic conditions. Dextrose, levulose and maltose are fermented by all types, proteins are digested, gelatin is peptonized, gelatin is liquefied and  $H_2S$  is produced. Indole is not formed and nitrates are reduced.

**Types.** Several types of *Cl. botulinum* are recognized by means of the immunological specificity of the several toxins. Of the five clearly recognized types, types A and B are largely responsible for disease in man, although a few outbreaks have been caused by Type E. Types C and D produce disease in animals. Type E is a widespread pathogen of birds responsible for duck sickness and

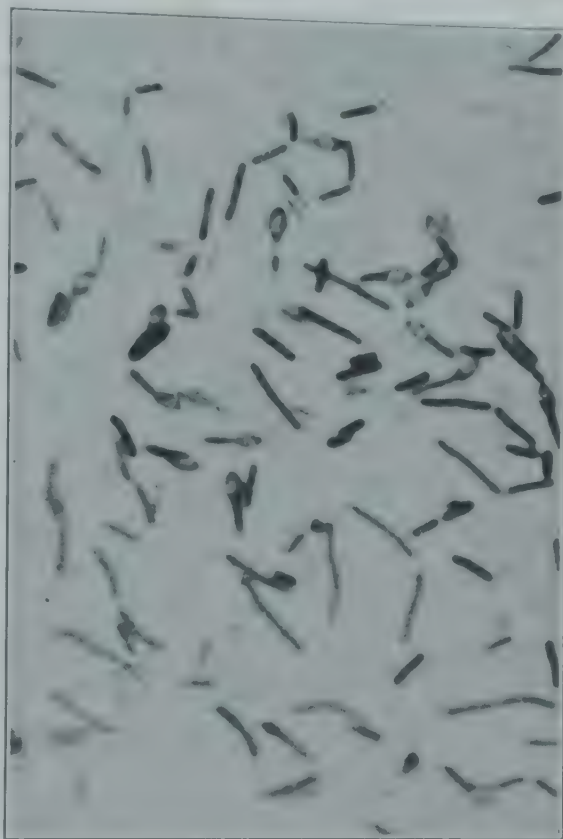


Fig. 151. Photomicrograph of smear from a pure culture of *Clostridium botulinum*. Note the sporulating rods and the poorly staining free spores. (Magnification approximately  $\times 1,400$ .)

"limberneck" of chickens; it is harbored by green bottle flies. Type D is associated with a paralytic disease of horses and cattle in South Africa. The strains of *Cl. botulinum* differ not only in that the type-specific toxins are separate immunological entities, but also in the fermentation of certain carbohydrates. In general, Types A and B are proteolytic, and Types C, D and E are non-proteolytic.

**Botulinum Toxin.** The pathogenicity of *Cl. botulinum* is due solely to its toxin, which in the natural disease is ingested preformed in foods, but which in laboratory animals is also active following injection. The washed vegetative bacterial cells are toxic but not invasive, whereas viable spores freed from the toxin may be injected in large numbers into animals without producing disease. Botulinum toxin has long been known to be protein in nature, heat labile, highly antigenic and remarkably resistant to the activity of enzymes of the digestive tract. It is the only true bacterial exotoxin which is active following ingestion. Recent crystallization of botulinum toxin Type A and Type B has been accomplished by means of chemical purification of acid-precipitable materials from crude cultures. This crystalline Type A toxin has the properties of a globulin, has a molecular weight estimated between 900,000 to 1,200,000 and behaves like a pure chemical substance. Chemical analyses and determination of the amino acid composition have not indicated the cause of the extreme toxicity. The crystalline Type A toxin contains approximately 32 billion mouse LD<sub>50</sub> doses per gram of material, a potency 240 times that of the crude culture. Toxoid may be produced by treating the toxin with formalin. The Type B toxin is also a protein with an estimated molecular weight of 60,000. It is highly toxic and differs from Type A toxin in its physico-chemical and serological properties.

The toxins of the several types all appear to act on the peripheral endings of both the autonomic and peripheral motor nerves to produce partial or complete paralysis of the innervated muscles. The activity is somewhat similar, but not identical, to that of curare, since paralysis seems to be caused by interference with acetylcholine synthesis. The sensory nerves are unaffected, and it is doubtful if any changes are produced in the nerve cell bodies. Although the several types appear to be identical with respect to the site of action, differences have been noted in the species of animals affected. Thus the rhesus monkey is resistant to Types C and D following feeding, and human intoxication with these types has not been reported; chickens are resistant to Type D toxin.

**Laboratory Diagnosis of Botulism.** The laboratory diagnosis of botulism depends upon the demonstration of botulinum toxin, preferably in suspected foods or stomach washings, but, at times, in necropsy tissue specimens or in cultures of bacteria isolated from food containers. Specimens of food and tissue should be thoroughly emulsified in saline, the mixture centrifuged and the supernatant fluid used to inject animals. Usually 0.5 ml. portions are injected intraperitoneally into mice, control animals receiving specific Types A and B antiserum in addition to the test material. Paralysis and death of the unprotected animals within four days constitutes a positive result. Guinea-pig feeding



may also be conducted. Cultivation of organisms is less reliable than the demonstration of toxin in suspected material because the organisms are so widespread in nature.

**Immunity.** Highly potent specific antitoxin may be produced against the different types of botulinum toxin by immunizing horses or rabbits. Purified antitoxin horse serum against both Types A and B is used in the treatment and prophylaxis of the human disease. A prophylactic dose of 5,000 units should be given for prevention and not less than 50,000 units for treatment of human tetanus. Formal toxoid is an effective immunizing agent for animals and has been used for the protection of laboratory workers. The disease is, however, too frequent to warrant use of the toxoid in the general human population.

## CLOSTRIDIUM TETANI AND TETANUS

Tetanus or "lockjaw" is a disease characterized by spasm (*i.e.*, severe protracted contraction) of the voluntary muscles which results from intoxication with the toxin of *Clostridium tetani*. The causative organism was isolated in 1884 by Nicolaier. Later Kitasato (1889) proved that the disease was caused by tetanospasmin toxin, and in 1890 von Behring and Kitasato demonstrated tetanus, as with diphtheria, antitoxin.

***Clostridium Tetani.*** The cells of *Cl. tetani* are slender (2–5  $\mu$  long and approximately 0.5  $\mu$  in diameter), motile, gram-positive rods with rounded ends. The spores are spherical, and their location at the end of the cells produces a "tennis racket" appearance. Colonies on blood agar are somewhat rhizoid in appearance and are hemolytic. The spores are highly resistant to heat and retain viability during storage for months or years. They also may remain viable in the tissues of the animal body for considerable periods of time.

*Clostridium tetani* grows well on ordinary bacteriological media under anaerobic conditions. Cultures develop best at 37° C, although growth occurs at higher and lower temperatures. Cultures may be maintained in deep meat or serum media. *Clostridium tetani* is relatively inactive metabolically in that it does not ferment any carbohydrates and is only moderately proteolytic. It utilizes a number of amino acids, liquefies gelatin and slowly attacks coagulated protein. Hydrogen sulfide and indole are produced.

*Clostridium tetani* is widely distributed in the soil and is commonly found in the intestinal tract of man and animals. From these sources it finds ready access to wounds.

**Pathogenicity.** *Clostridium tetani*, like the organisms of gas gangrene, is not only the cause of wound infections in man; it does not invade the tissues of the body and does not infect through intact epithelial surfaces. It is able to multiply in tissues which, because of mechanical or chemical injury or the growth of other microorganisms, provide suitable conditions of anaerobiosis. In addition to infection of wounds, tetanus may result in infants from contamination of the umbilical stump (*tetanus neonatorum*), and may develop following smallpox

vaccination with contaminated virus or when the vaccination area has been tightly covered with contaminated shields. Natural infection of animals, particularly horses, is known, and the disease may be reproduced in mice, pigs, rabbits, rats, goats, horses and monkeys. Dogs and cats are less susceptible.

Tetanus results from intoxication with the exotoxin produced by *Cl.* In man the incubation period may vary from a few days to several weeks, even in experimental animals receiving large doses of toxin the onset of symptoms is delayed several hours.

**Tetanus Toxin.** Filtrates of cultures of *Cl. tetani* contain two toxic substances, **tetanolysin**, an hemolysin which does not reproduce the disease, and **tetanospasmin**, which upon injection will give rise to symptoms of tetanus. Tetanospasmin is a true bacterial exotoxin elaborated during growth of the organisms either in tissues or in suitable culture media. The toxin is proteinaceous, heat labile, and highly antigenic. Recently, report has been made of the purification of toxin by precipitation with methyl alcohol under controlled conditions; the material contains between 50,000,000 and 75,000,000 mouse units per milligram of nitrogen and gives positive tests for protein.

As indicated above, the result of tetanus intoxication is a spasmodic contraction of the voluntary muscles. The effects may be localized to the muscles of an injected extremity or they may be progressive and generalized, affecting many muscles of the body. The site and mode of action of the toxin are poorly understood and are controversial. Evidence has been presented, particularly by Meyer and Ransom, that the toxin is absorbed at the myoneural junction in the area of infection, passes to the central nervous system via the axis cylinder of the nerves and produces its effect only after reaching the motor nerve cells (anterior horn cells). Such a theory is supported by the development of tetanus first in an injected limb, by demonstrated toxicity of the nerve from an injected limb, by unilateral inhibition of tetanus with locally injected antitoxin. If the toxin is injected bilaterally, by the long incubation period and by producing only generalized tetanus following intravenous injection of toxin. The second theory is based upon evidence that tetanus toxin is disseminated throughout the body in the lymph and blood and hypothesizes that tetanospasmin acts locally on the myoneural junction to produce local tetanus and also centrally to give rise to generalized tetanus. This point of view is based upon demonstration of toxin in lymphatic tissues and blood draining an injected limb and failure to produce tetanus by direct injection of a nerve (sciatic) trunk. Although the first theory is probably more widely accepted, present evidence does not permit a decision as to the correct view. It is agreed, however, that tetanus toxin produces muscle spasm by action upon the motor nervous system.

**Toxoid.** Formalinized tetanus toxoid may be used for immunization of humans and animals. Alum-precipitated toxoid appears to be the material of choice. One milliliter quantities of a potent preparation are given at intervals for several injections, and a stimulating dose may be given yearly or upon suspicion of infection. The toxoid has now been widely used in the armed forces and is



ended for children. Its use precludes the necessity for prophylactic anti-

**Tetanus Antitoxin.** Tetanus antitoxin is produced commercially by the immunization of horses; that prepared for human use is a purified antitoxic serum. Tetanus antitoxin is standardized in terms of its protective power against toxin. Several units have been described; the international unit is an arbitrary unit representing the neutralizing activity of c.1547 mg. of the standard toxin. The American unit, which is 10 times the amount of antitoxin which protects a 350 gm. guinea pig for 96 hours against a standard dose (approximately 100 MLD) of toxin, is about twice the international unit. The flocculation reaction with tetanus toxin-antitoxin mixtures is less satisfactory than in the case of diphtheria.

For the prevention of tetanus in man, 1500 units of antitoxin should be given to unimmunized persons who have contaminated, lacerated wounds. In treatment of the disease 10,000 or more units are used. Good results have been obtained by the use of tetanus antitoxin in the prophylaxis and treatment of the disease in animals and, although comparable figures are not available for man, the disease is highly fatal even with antitoxic therapy, antitoxin appears to have some therapeutic value. Surgical and other necessary treatment should, of course, be given.

## **THE NONSPORE-FORMING ANAEROBIC BACTERIA**

Numerous bacteria which grow only in the absence of oxygen and which do not form resistant spores have been described since the early days of bacteriology. Nonsporulating anaerobic bacteria may be isolated from the nasopharynx, the intestinal tract and the female genital tract of normal individuals. As a group, however, they tend to be associated with pyogenic or necrotizing infections of the mucous membranes. These infections frequently follow tissue injury or may develop in the presence of lowered resistance, such as that associated with dietary deficiency. In many instances predisposing factors to infection are, however, absent. The infections tend to progress slowly and may be characterized by the presence of foul-smelling pus or membranous inflammation. Healing may be slow, and spread from the initial site is not uncommon. Infrequently septicaemia occurs and abscesses develop in parts of the body distant from the initial site of infection. Such generalized infections are often fatal in a few days or weeks.

The nonspore-forming anaerobic bacteria are most often found in mixed infections, in which two, three or more members of the group are found in close association with each other or with aerobic organisms. Such is the case, for example, in infections about the teeth (periodontitis or pyorrhea alveolaris), in the infection of the mucous membranes of the mouth and pharynx, in peritonitis following ruptured appendix, lung abscess and often puerperal infection. In general, the virulence of mixed infections is greater than that of pure

cultures of the organisms. On the other hand, pure cultures may be from abscesses or from the blood and are capable of producing severe disease. The virulence of different strains of the same organism is not the same, some strains producing severe infections in laboratory animals whereas others is followed by formation of small local abscesses or no visible infection. Virulence is quickly lost during cultivation in the labor

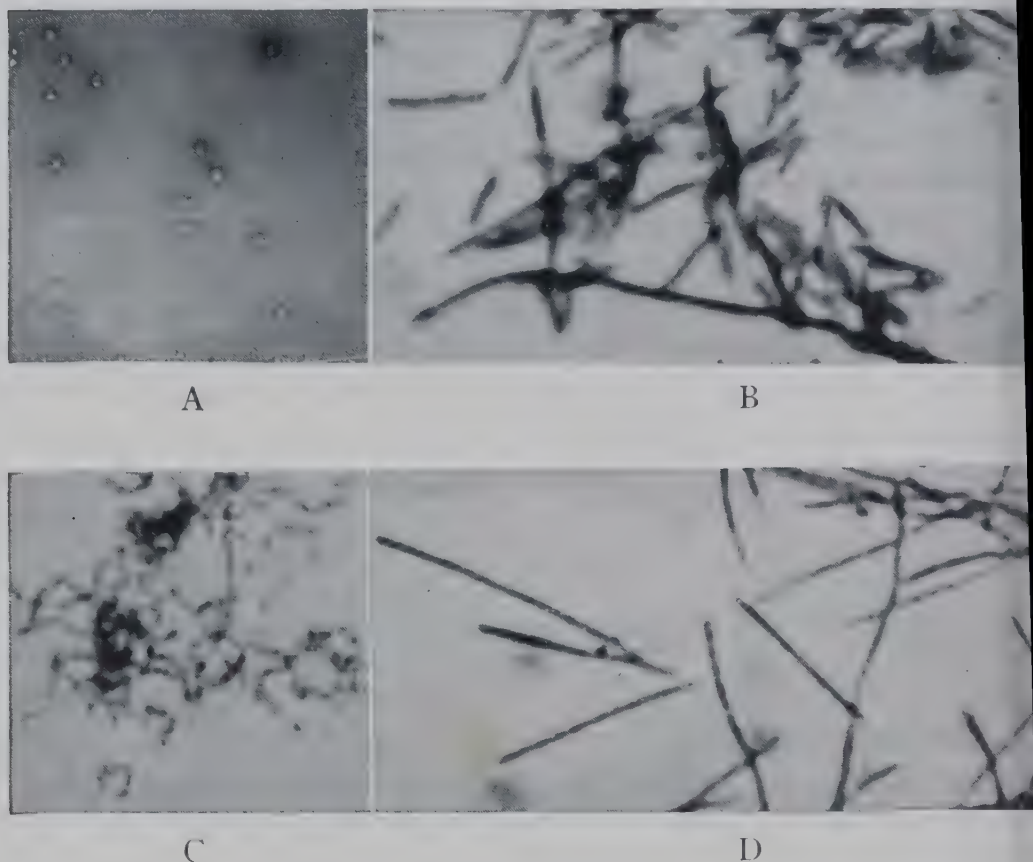


Fig. 152. Nonsporulating Anaerobic Bacteria. (A) Colonies and (B) smear of *Bacterium necrophorum*, (magnification approximately  $\times 2,700$ ); (C) smear of anaerobic streptococci, (magnification approximately  $\times 1,000$ ); (D) smear of fusiform bacteria, (magnification approximately  $\times 2,700$ ). (Courtesy of Dr. G. M. Dack.)

general, infection with nonsporulating anaerobes does not seem to be transmissible to normal individuals, and development of disease appears to depend on endogenous infection in individuals with lowered resistance.

The bacteria of this group have in common the inability to grow in the presence of atmospheric oxygen and the production of similar types of infections. They are a little known, but none the less medically important, group of bacteria. According to the systems for classification of bacteria they should be included in several different genera and families. However, it is felt that they are sufficiently similar to be considered together.

**The Anaerobic Streptococci:** Morphologically the anaerobic streptococci form chains as do the aerobic organisms of this group. Some produce gas



from culture media, particularly if fresh animal tissue or blood is used. Others of the group are relatively inert physiologically. The members of the group are distinguished and classified with some difficulty in the laboratory. The organisms of importance in human disease are usually classified as *Str. foetidus* or *Str. putridus* and *Str. putridus*. The first two organisms are similar if not identical. *Streptococcus putridus* seems to be different from the other two. These cocci produce gas in culture media under suitable conditions and are found in foul-smelling, necrotic lesions in man. Anaerobic streptococci have come to be associated particularly with local or generalized infections following childbirth, i.e., puerperal fever. They may also be isolated from other infectious processes and from normal individuals.

**The Fusiform Bacteria.** The fusiform bacteria (*Fusobacterium spp.*) are spindle-shaped, gram-negative bacteria with pointed ends (Fig. 152). They grow poorly on laboratory media. These organisms are commonly found in lesions of the mucous membranes, such as Vincent's stomatitis, in putrid lung abscesses and other mixed infections. The pus from such abscesses is foul-smelling. On a smear it may be seen to contain large numbers of fusiform rods, spiral bacteria, shorter rods and cocci. Like the anaerobic streptococci, fusiform bacteria have been isolated from the normal mucous membranes of the throat.

**Bacterium (Bacteroides) Funduliformis or Bacterium Necrophorum.** This organism has been found in the genital tract of patients ill with puerperal infection and of normal individuals, in purulent infections of the mucous membranes of man and animals, in abscesses of the lung and other tissues and in blood. Some strains are highly virulent for laboratory animals, whereas others are avirulent. Morphologically this organism is a bizarre, highly pleomorphic, gram-negative rod (Fig. 152). Cultures have a putrid odor, and in liquid media gas is formed.

**Bacterium (Bacteroides) Melaninogenicum.** This bacterium is a small, gram-negative rod, cultures of which on media containing fresh blood are completely black or deep brown. Like the other nonsporulating anaerobes, *Bacteroides melaninogenicum* may be isolated from normal mucous membranes but is more commonly found in necrotic and purulent lesions or other infections. It is commonly found in association with other members of the group.

**Anaerobic Staphylococci.** Anaerobic cocci which grow in clusters have been described. Of these bacteria, *Staphylococcus parvulus* is the best known. It may be found in abscesses and peritonitis following appendicitis. The organism is a small, gram-negative, strictly anaerobic coccus which grows in chains and produces an abundance of putrid gas. By suitable methods an ectoplasm of amorphous material may be demonstrated about the cells. It is slightly pathogenic for animals.

**Bifidobacteria and Vibrios.** Strictly anaerobic, fastidious, curved and spiral bacteria may be found in the normal mouth and in necrotic lesions of the mucous membranes. As a whole the group has been incompletely studied and their

significance in disease production remains in doubt. Spiral organisms (and spirochetes) are commonly found in Vincent's infection.

**Nonpathogenic *Bacteroides*.** When specimens from the intestinal contents of man and animals are examined by both aerobic and anaerobic bacterial methods, it is frequently observed that the anaerobic bacteria outnumber aerobic organisms. The nonsporulating, anaerobic, rod-shaped bacteria constitute the predominant organisms. These bacteria, which are both gram-negative and gram-positive organisms, have been classified into many different genera and species. As a group they are often referred to as *Bacteroides*, although the generic name is sometimes reserved for the gram-negative, pathogenic anaerobic species, the others being included in *Corynebacterium*, *Dialister*, etc. The significance of the nonpathogenic members of the group is unknown. Some appear to be intestinal parasites. Some are proteolytic, whereas others are said to digest proteins. Fermentation of sugars is different in different species. Differentiation of members of *Bacteroides* is laborious and uncertain, so that only a few publications and detailed classifications of bacteria should be consulted for further information.



# 30

## THE GENUS CORYNEBACTERIUM— DIPHTHERIA

The *Corynebacteria* are parasitic, aerobic, nonmotile, slender, slightly curved b-shaped rods which stain irregularly with the usual bacteriological dyes. The organisms are gram-positive and, when suitably stained, may have a segmented or barred appearance. The most important member of the genus is *Corynebacterium diphtheriae*, or the diphtheria bacillus, which is the cause of diphtheria. Although diphtheria had been known as a clinical entity for many years, the diphtheria bacillus was first observed by Klebs in 1883 and was cultivated by Löffler in 1884 from cases of diphtheria and from the throats of healthy carriers. The exact relationship of the diphtheria bacillus to the disease remained in doubt, however, until Roux and Yersin in 1890 were able to reproduce the characteristic symptoms and lesions of diphtheria by the use of the soluble toxin produced by *C. diphtheriae*. In 1890 von Behring and Kitasato first demonstrated unequivocally the importance of antibodies in immunity to infectious diseases when they demonstrated the effectiveness of diphtheria and tetanus antitoxins.

In addition to the diphtheria bacillus, the genus *Corynebacterium* includes important parasitic organisms of man such as *C. jeikeium* and *C. xerosis*. The latter, which was the first member of the genus to be isolated, has been cultivated from the normal conjunctiva and is probably a cause of conjunctivitis. Furthermore, a number of important animal pathogens are members of this genus. Thus *C. ovis*, or the Preisz-Neisser bacillus, is a natural pathogen of horses, sheep and sometimes cattle and *C. burnetii* is a cause of purulent or suppurative infections in cattle.

**Morphology.** The genus name *Corynebacterium* is derived from the typical morphology of these organisms. The morphology is perhaps best illustrated by that of the diphtheria bacillus, which is a slender rod approximately



Fig. 153. Colonies of *Corynebacterium diphtheriae* on blood agar.

1 to 6  $\mu$  in length. The individual cells are typically straight or curved shaped rods, although branched and Y or V shapes may be seen in older cultures. This bizarre morphology, together with the characteristic arrangement of individuals in packets of parallel cells resembling palisaded cigars, is the typical morphological appearance to stained smears of these organisms.

Because of the presence of granules (known as Babes-Ernst or metachromatic granules), the cells stain irregularly, giving a barred or granular appearance to the rod. The irregular staining, which is poorly seen in preparations stained by Gram's method, is clearly apparent in smears stained with Löffler's alkaline methylene blue or other special dyes where the granules are stained more deeply than the rest of the cell.

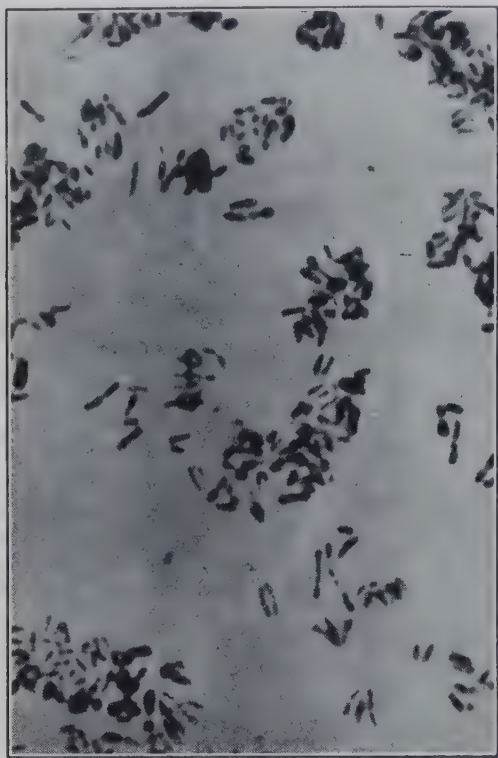


Fig. 154. Smear of *Corynebacterium diphtheriae*. Note the deeply staining granules within the cells and the typical parallel arrangement of the cells. (Photomicrograph magnification approximately  $\times 1,400$ .) (Kral.)

Although the diphtheria bacillus is, in general, a more delicate appearing organism and is more granular than other members of the genus, the morphological appearance is insufficient for identification of this organism. On the other hand, the observation of morphologically atypical organisms in smears of cultures from the throat of persons suspected of having diphtheria or from normal individuals is an important step in the identification of the diphtheria bacillus. The *Corynebacteria* do not possess flagella and hence are nonmotile. They do not retain the Gram stain. Because of the polymorphism of members of this group, the observation of Y-shaped, V-shaped, or branched forms, *Corynebacteria* are often confused with the Actinomycetales rather than with the Eubacteriales, or true bacteria.

On Löffler's coagulated serum medium (heat-coagulated, blood, and dextrose broth) or on blood agar, colonies of the diphtheria bacillus are approximately 1 mm. in diameter, gray, entire or slightly irregular and somewhat elevated. Stained smears from growth on Löffler's medium reveal morphologically typical organisms, so that this medium has been widely used for the identification of diphtheria bacilli. On potassium tellurite medium (chocolate agar containing 0.5 per cent potassium tellurite), the colonies of the diphtheria bacillus have a typical appearance, which has value in the recognition of *C. diphtheriae* and differentiation of types. Because of reduction of the tellurite ion, the colonies become grayish or jet-black in color.

Smooth to rough variation occurs in the diphtheria bacillus and is a



loss of virulence, but not necessarily with a loss of ability to produce toxin. For S, S  $\rightarrow$  R or R strains of the organism are capable of producing toxin. In addition to the smooth and rough variation, mucoid and dwarf colonies have been described for *C. diphtheriae*, and the *gravis*, *mitis* and *intermedia* types of this organism (see below) are associated with differences in colonial morphology. The latter differences are apparently related to the S  $\rightarrow$  R variation.

**Physiology.** The *Corynebacteria* develop at both room and body temperatures, the latter being optimum. They grow rapidly on suitable media, good growth being obtained within 24 hours' incubation. Members of the group are readily destroyed at 60° C or by boiling temperatures. They are, however, able to survive in the laboratory on artificial media for many weeks or months. It is interesting that these organisms are somewhat sensitive to acid, and that an alkaline reaction, approximately pH 8, is most satisfactory for growth. Although they are usually considered to be aerobic organisms, oxygen is not an absolute requirement and some strains will develop under completely anaerobic conditions. *Corynebacterium diphtheriae* does not liquefy gelatin or attack coagulated proteins. Indole and nitrites are not produced; fermentation of dextrose and glucose occurs with the production of acid (lactic, succinic, formic, acetic, and pyruvic acids) but not gas with all strains; and the majority of cultures ferment maltose, galactose, dextrin and glycerol. It is important that sucrose, mannose and mannitol are not fermented by *C. diphtheriae*. The pseudo-diphtheria bacillus (*C. hojmanni*) attacks no sugars, and *C. xerosis* ferments dextrose, maltose, and sucrose with the production of acid, but it does not ferment dextrin or mannitol. Other *Corynebacteria* also differ from the diphtheria bacillus in their characteristic metabolic reactions. However, the metabolic characteristics of the organism do not suffice to identify *C. diphtheriae* because of variations in its characteristic reactions.

The requirements of the diphtheria bacillus for growth and for toxin production have been studied extensively. Although growth is most luxuriant and characteristic on media containing blood, blood serum or other body fluids, these substances are not required for growth of *C. diphtheriae*, and development occurs on ordinary laboratory media. *Corynebacterium diphtheriae* does not grow on chemically defined media in the absence of added vitamins or growth factors. All strains uniformly require the presence of a number of amino acids and the majority require the growth factors pimelic acid, nicotinic acid and beta-alanine. However, pimelic acid may be replaced by biotin, so that it appears that pimelic acid is utilized by the organism in the synthesis of biotin. Similarly, beta-alanine may be replaced by pantothenic acid for some strains.

In addition to the amino acids and vitamins, traces of numerous elements, such as magnesium, calcium, iron, manganese, zinc and copper, are required for growth of *C. diphtheriae*. It has been found that the concentration of salts of these elements is extremely important, minimal amounts being maximum for growth in production, although not necessarily maximum for growth of the organism. The growth requirements of other *Corynebacteria* are poorly known.

**Types of the Diphtheria Bacillus.** Study of the bacteria isolated in years from diphtheria of particularly fatal, mild or intermediate severity led to the recognition of three types of *C. diphtheriae* known as *gravis*, *mitis* or *intermedius*, respectively. Although infections with the *gravis* type in general have a higher case fatality than those caused by the *mitis* or *intermedius*, these relationships are not constant, and *mitis* or *intermedius* infections may be highly fatal. Experimentally a high proportion of strains of all three types are virulent for animals. Diphtheria toxin produced by all types is neutralized by the same antitoxin. However, *gravis* strains are reported to produce a second factor which has the properties of a spreading factor and which appears to increase the virulence of diphtheria infections. Although the association of particular types of *C. diphtheriae* with the malignant and mild forms of diphtheria is inconstant, there is little doubt that certain European epidemics of particularly severe and relatively antitoxin-resistant forms of the disease have been related to prevalence of the *gravis* type. In the United States we have been relatively free of this severe diphtheria.

Types of *C. diphtheriae* differ morphologically and culturally. Thus on a nutrient medium, colonies of the *gravis* type are large, irregular in outline, striated and gray in color; those of the *mitis* type are small, smooth and black in color; *intermedius* type colonies are flat, with a black center and gray border. In stained preparations cells of the *gravis* type are short, thick rods which stain uniformly; *intermedius* type are long, slender, often clubbed cells which have a barred appearance; cells of the *mitis* type are slender rods with prominent metachromatic granules. *Gravis* strains characteristically ferment starch and glycogen, whereas *mitis* and *intermedius* strains do not attack these substances. *Mitis* strains are distinctly hemolytic. The diphtheria bacillus types also differ antigenically.

**Pathogenicity.** The Corynebacteria are pathogens of man and animals, the most important of which is, of course, *C. diphtheriae*. However, several other members of the genus have been associated with naturally occurring diseases in animals and have been shown to produce toxin. Thus *C. ovis*, or the Pasteur Nocard bacillus, is the cause of lymphadenitis in sheep and horses. The disease is primarily a purulent or abscess-producing infection, although the organism also produces a filtrable toxin. Similarly, *C. pyogenes* has been isolated from various septicemic infections in cattle and other animals.

Of the parasitic Corynebacteria, *C. diphtheriae* and *C. ovis* are toxigenic, whereas toxin production by *C. hoffmanni*, *C. pyogenes* and others has not been demonstrated. Indeed, the pseudo-diphtheria bacilli, *C. hoffmanni* and *C. xerosis*, are only questionably pathogenic. Experimentally, guinea pigs and rabbits are particularly susceptible to diphtheria toxin, whereas rats and mice are extremely resistant and other laboratory animals, including a number of birds, are moderately susceptible. Similarly, *C. ovis* and *C. pyogenes* are pathogenic for guinea pigs and rabbits and are less so for other laboratory animals. The little known *C. murium*, which is naturally pathogenic for mice is, on the other hand,



nonpathogenic for other laboratory and domestic animals. It should be noted that the toxin formed by *C. ovis* is not neutralized by diphtheria antitoxin *vice versa*.

## DIPHTHERIA

Diphtheria is an acute communicable disease caused by *C. diphtheriae* and characterized by the presence of throat infection with pseudomembrane formation and a general intoxication with diphtheria toxin. The disease usually begins two to seven days after infection with the microorganism. The diphtheria bacillus remains localized in the pseudomembrane, which is composed of an inflammatory exudate consisting of fibrin, many leucocytes and other inflammatory cells, together with the diphtheria bacilli. The site of infection is most often in the nasopharynx in the region of the tonsils, but it may be located in the nose (nasal diphtheria) or in the larynx, the trachea and the bronchi (laryngeal diphtheria). The site of primary infection is rarely located in other mucous membranes, although diphtheria infection of wounds and of the eye are occasionally encountered. Ordinarily the diphtheria bacillus does not invade the tissues widely from the initial site of the infection. However, it occasionally may be isolated from the deeper tissues or from the blood.

The lesions of diphtheria are, thus, in part due to the presence of the bacterial local lesions but, of greater importance, toxin produced by the bacteria is absorbed and produces injury in many tissues and organs of the body. In man the toxin is particularly injurious to the heart muscle, the nervous system and the kidneys, producing acute heart failure, degeneration of the kidneys and paralysis. In animals diphtheria toxin characteristically gives rise to hemorrhages in the adrenal gland, an effect which is inconstantly observed in human diphtheria. Experimental animals seldom develop diphtheria with pseudomembrane formation following inoculation of the mucous membranes. On the other hand, subcutaneous inoculation with either cultures or filtrates of cultures results in the production of hemorrhage and edema at the site of the inoculation, typical hemorrhages in the adrenal glands, and degenerations of the cardiac muscles.

**Diphtheria Toxin.** The virulence of the diphtheria bacillus is related almost entirely to its ability to produce potent exotoxin. Diphtheria toxin is produced in the laboratory by growing the microorganisms for 7 to 10 days in a suitable liquid broth containing small amounts of iron and other minerals. The organisms are then removed from the culture by centrifugation and filtration through bacteriological filters. The resulting clear culture liquid, free of microorganisms, constitutes crude toxin. Such crude toxic filtrates, in amounts of 0.001 ml., are sufficient to kill a guinea pig.

Diphtheria toxin is a typical exotoxin in that it is protein in nature, is rapidly destroyed by heat and proteolytic enzymes and is highly antigenic. It is stable in alkaline solutions but is rapidly destroyed by acid. Purification studies have resulted in the separation of a protein which possesses all of the properties of diphtheria toxin. This protein, which is composed of the usual amino acids, has

a molecular weight of approximately 72,000 and is fatal to the guinea pig in amounts of 0.001 mg. The relationships of diphtheria toxin to natural and experimental diphtheria are shown by the reproduction of typical pathological changes in experimental animals by inoculation of crude or purified diphtheria toxin, by the ability of specific antitoxin against this material to protect animals from experimental disease and to alleviate the symptoms and findings of diphtheria in man and by the protective effect of active immunization against subsequent infection with *C. diphtheriae* in man and animals. Diphtheria toxin may be detoxified by treatment with formalin to produce toxoid which may be used for active immunization of man and animals.

Diphtheria toxin is standardized by means of virulence tests in animals. In guinea pigs the minimum lethal dose (MLD) is determined by the subcutaneous inoculation of a series of guinea pigs with diphtheria toxin. The minimum lethal dose of diphtheria toxin is defined as that amount of toxin which, on the average, will kill a 250-gram guinea pig on the fourth day following subcutaneous inoculation (Chapter 22). A second test for the potency of diphtheria toxin is one in which the toxin is injected into animals in combination with one unit of standard antitoxin (see Chapter 23). In this test the L<sub>50</sub> dose of toxin, or that amount which in combination with one unit of standard antitoxin will on the average kill a 250-gram guinea pig on the fourth day, is used. The standardization of diphtheria toxin is important in determining the amount of toxin to be injected in the Schick test and in the standardization of lots of diphtheria antitoxin.

**Determination of Infection and Immunity in Diphtheria.** The diagnosis of diphtheria depends upon both clinical and laboratory findings. In the laboratory, material from the nose and throat of suspected cases of diphtheria is cultured on Löffler's serum medium or on tellurite medium. After 18 to 24 hours of incubation the cultures are observed grossly and microscopically for typical *diphtheriae*. In the presence of symptoms of diphtheria, the demonstration of a culturally and morphologically typical *C. diphtheriae* is usually sufficient to confirm the diagnosis. However, it is important to determine the toxicity and virulence of cultures isolated from the nose and throat of normal carriers of infection. In the usual virulence test, cultures or filtrates of cultures of suspected *C. diphtheriae* are inoculated into guinea pigs. If the inoculated culture is virulent, the susceptible guinea pig should die within a few days with lesions typical of diphtheria, whereas a second guinea pig protected with diphtheria antitoxin should be unaffected by the inoculation. In a second test, known as the Römer test, small amounts of cultures are inoculated intracutaneously into a normally susceptible guinea pig and into a guinea pig protected with diphtheria antitoxin. Because the toxin of the diphtheria bacillus produces a cutaneous reaction, virulent cultures give rise to reddened, necrotic areas in the skin of the unprotected, but not in that of the antitoxin-protected, guinea pig. The virulence test should not be omitted in the examination of cultures isolated from suspected carriers, since pseudo-diphtheria bacilli are morphologically similar and infection with the latter organisms does not require isolation or treatment. Direct examination



of materials from the pseudomembrane in diphtheria is time consuming and is not sufficiently satisfactory to replace the cultural methods or animal tests, although it may be helpful and serve to detect Vincent's infection. Susceptibility and immunity in diphtheria are determined by means of the Schick test, which is performed by injecting 0.1 ml. of toxin containing 1/500 of diphtheria toxin into the skin of the forearm. A second injection of the same medium or heat-inactivated toxoid is made in order to detect hypersensitivity to ingredients of the medium. In the susceptible individual an area of redness, swelling or induration, and sometimes necrosis, similar to that observed in animals in the Römer test, appears within 48 to 72 hours at the site of injection of the diphtheria toxin. In the immune individual, on the other hand, no reaction appears. In case of hypersensitivity to ingredients of the medium from which the toxin has been produced, an equal reaction will appear at both sites of injection. A negative reaction to the Schick test results from the presence of antitoxin in the blood and for this reason it is correlated with resistance. It was formerly estimated that a negative reaction to the Schick test indicated 1/20 to 1/40 unit of available antitoxin. However, present evidence suggests that the amount is only a fraction of this figure, probably less than 1/250 unit. The amount of antitoxin is, none the less, usually sufficient to protect against ordinary exposure, although following massive exposure persons who react negatively to the Schick test may develop diphtheria. In such instances, however, the disease is usually mild.

**Epidemiology.** With the exception of a few isolated geographical regions, *C. diphtheriae* is world-wide in distribution and is found in tropical as well as temperate zones. The disease is, however, more frequently recognized in temperate climates, whereas the carrier rate is reportedly higher in many tropical areas. In the United States 13,000 to 17,000 cases of diphtheria are reported annually, although the number may be as great as 100,000 in epidemic years. The disease is more prevalent during the winter months in temperate climates. The natural habitat of the diphtheria bacillus is the respiratory passages of man and organisms are disseminated from person to person principally by direct contact and air-borne infection, although milk-borne outbreaks of disease sometimes occur. The bacilli are present in large numbers in the discharges from the nose and throat of patients, but they are also encountered in latent infections in immune carriers and susceptible individuals. Infection may thus be acquired by direct contact with patients or with healthy carriers.

Extensive study has been made of the development of immunity against diphtheria and of the dissemination of *C. diphtheriae* within the population. By means of the Schick test, it has been found that a large proportion of children less than six months of age are resistant to diphtheria. This immunity, which is congenital passive immunity, is short-lived, so that preschool and early school age children are highly susceptible. Thereafter the proportion of immunes progressively increases until approximately 80 per cent of adults are Schick negative. The immunity acquired during childhood and adolescence is active

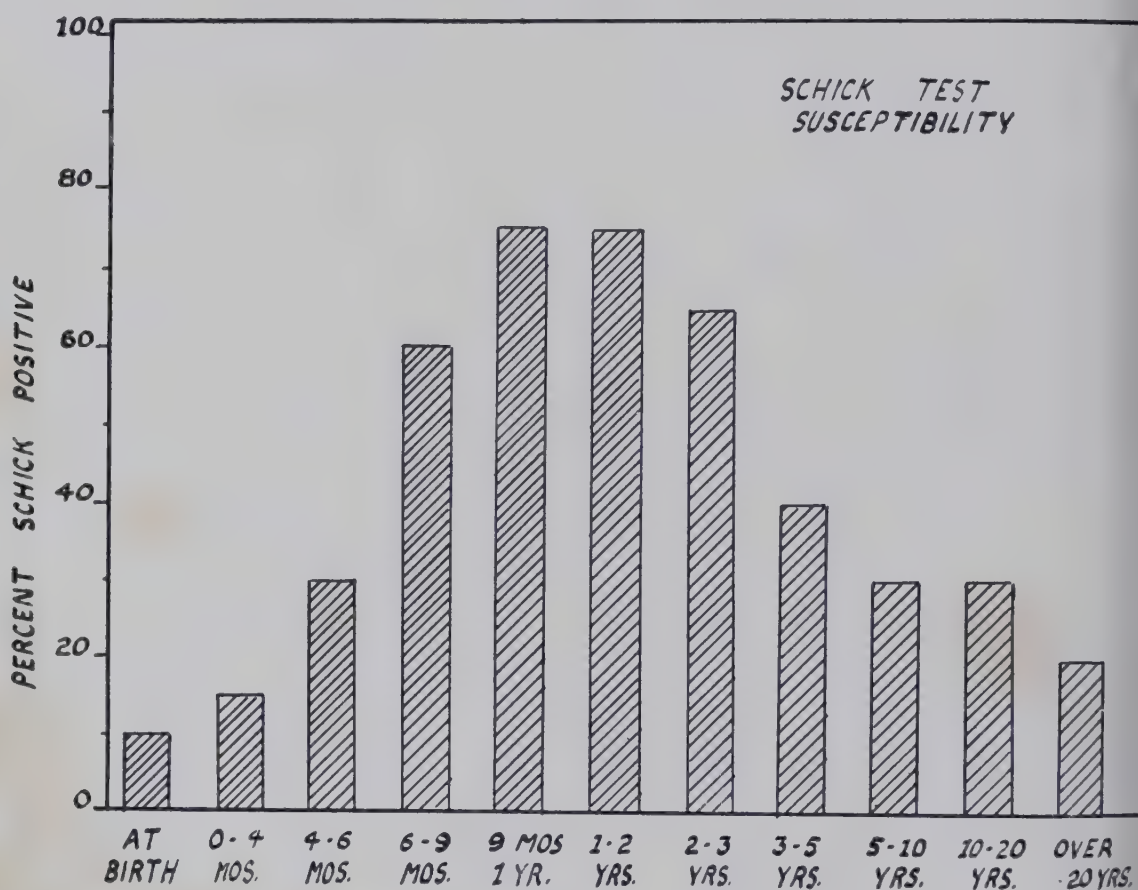
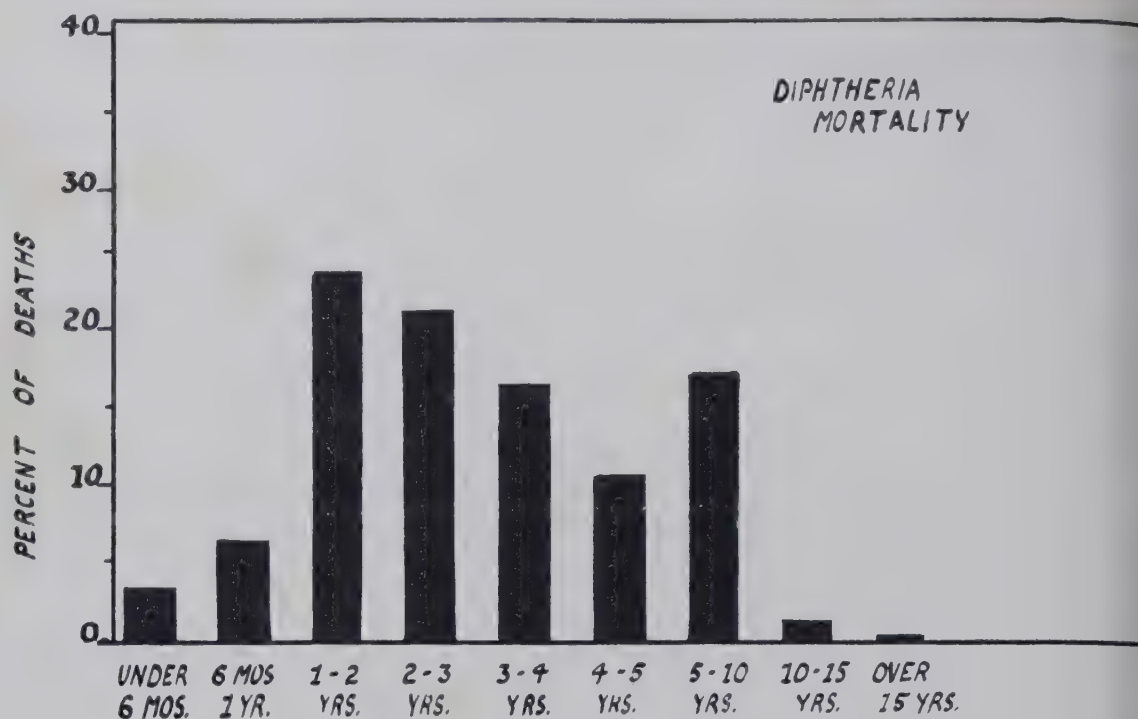


Fig. 155. Age distribution of susceptibility and mortality in diphtheria. (Data from Rosenau: *Preventive Medicine and Hygiene*, D. Appleton-Century Co., Inc.)



immunity resulting naturally from clinical or subclinical infection with *C. diphtheriae*, although artificial immunization with toxoid also results in active immunity. The relationship of susceptibility to diphtheria, as determined by Schick test and mortality from the disease, is shown in Fig. 155. Natural immunity is acquired at an earlier age in urban than in isolated rural communities and is more prevalent in tropical than in temperate regions.

The acquisition of natural immunity suggests a wide distribution of *C. diphtheriae*, a fact which is amply confirmed by cultural studies. The carrier rate has been estimated to vary from less than 1 to 5 per cent and to be higher among persons having close contact with patients. Carriers are more often temporary than chronic and *C. diphtheriae* is readily spread within a short time from one to another individual of the population. The identity of individual carriers is thus constantly changing. The opportunities for infection rise with school attendance, and so do the number of cases and the degree of immunity from small repeated infections. The case fatality is, however, higher among preschool children.

**Immunity.** Convalescence from diphtheria provides a high degree of resistance to a second attack of the disease. Immunity in diphtheria, whether the result of a clinical attack of the disease, congenital passive immunity, subclinical infection or artificial immunization, is an antitoxic immunity, and is dependent on the presence of antitoxin in the blood, other body fluids and tissues. The antigenicity of diphtheria toxin and toxoid has provided the basis for the artificial active immunization of man and animals and for the production of diphtheria antitoxin for passive immunization.

**Passive Immunity.** Passive immunity occurs naturally as congenital passive immunity or is produced by the injection of antibody, that is, antitoxin in the form of the refined serum of hyperimmunized animals, into susceptible individuals. Passive immunity finds its greatest use in the treatment of cases of diphtheria, although antitoxin in small doses may be used for the protection of susceptible exposed persons. Commercial diphtheria antitoxin is produced by the infection of horses with progressively increasing amounts of diphtheria toxoid and toxin until a satisfactory titer of antitoxin is produced. The animal is then bled, usually from the external jugular vein, the blood serum is separated from blood cells, and the antitoxin is partially refined and concentrated by chemical methods.

**Standardization of Diphtheria Antitoxin.** The reactions of diphtheria toxin and antitoxin have been discussed in Chapter 23. Since the combination of these substances is so important to immunity in diphtheria, the practical aspects of the reactions are repeated here. The combination with antitoxin results in a quantitative neutralization or inhibition of the ability of toxin to damage the animal body. This fact is utilized in the standardization of antitoxin by the neutralization or protection test in guinea pigs. In this test, the protective powers of a standard and an unknown antitoxin are compared against the same toxin. Thus the amount of the unknown antitoxin which has the same protective power as one unit of the standard antitoxin also contains one unit of antitoxin.

Originally the antitoxin unit was defined as that amount of antitoxin which would protect a guinea pig against 100 MLD of toxin; a unit which was replaced by the arbitrarily chosen International Antitoxin Unit because of the differences in the combining power of different toxins and, hence, differences in potency of antitoxins. Each unit of antitoxin, therefore, now possesses the same combining power as that of the standard antitoxin. Antitoxin may also be standardized by means of the Römer skin test (see virulence tests), since neutralization of toxin prevents development of the skin reaction. In addition, the flocculation or Rasth test, which depends upon the formation of a precipitate when equivalent amounts of toxin and antitoxin are mixed in the test tube, may be used.

**The Use of Antitoxin.** In the treatment of diphtheria, 10,000 to 100,000 units of antitoxin are commonly given intramuscularly, although intravenous injection may be used. In general, smaller doses are required for early and mild diphtheria and larger amounts are used when treatment is delayed or the disease is severe. The value of antitoxin in treatment of diphtheria is unquestionable and its administration should not be postponed after the diagnosis is made, since recovery is more certain following early use of antitoxin. One or more injections of antitoxin may be given after suitable tests for hypersensitivity to horse serum.

Antitoxin has been employed in small doses (2,000 to 5,000 units) for the prevention of diphtheria in exposed susceptible persons. The protection afforded is of only a few weeks duration and is followed by a return of susceptibility and by an equally undesirable hypersensitivity to horse serum. Antitoxin is, therefore, infrequently used for prevention and then only in young children who have received massive exposure, in the presence of complicating illness or in other unusual circumstances. More durable protection is afforded by active immunization.

**Active Immunization.** Active immunization is notably successful in the prevention of diphtheria and has become a highly important part of preventive medicine and pediatrics. Preventive immunization is best carried out at the period of life when congenital passive immunity is disappearing and before the susceptible child has received massive exposure to infection, *i.e.*, in the second six months of age. Protection is thus provided during the highly susceptible preschool years when the fatality rate from diphtheria is maximum. However, active immunization may be carried out at any age, and preschool and school-age children are immunized with good results. Because of the increased exposure to infection which begins with school attendance, it is particularly desirable to protect children before this time. Immunization may, indeed, be required for school attendance. Active artificial immunization confers a high degree of protection against diphtheria which is comparable to that provided by recovery from the disease. Immunity is manifest by a negative reaction to the Schick test. The protection is durable and usually only one immunization is necessary, although the procedure may be repeated if necessary and a single stimulatory injection of toxoid is frequently given to immunized children prior to school attendance.

The first preparation used extensively for active immunization was a mixture



toxin-antitoxin. Injection of toxin-antitoxin, which consists of a slightly under-neutralized toxin, is followed by the slow release of toxin, thus providing a continuous antigen (toxin) to stimulate formation of antitoxin. The amount of toxin liberated at any one time from a satisfactory preparation is insufficient to produce intoxication. Although successful in a high proportion of cases, toxin-antitoxin immunization has largely been replaced by the slightly more potent and generally safer toxoid preparations.

**DIPHTHERIA TOXOID.** Diphtheria toxin incubated with formalin until non-toxic was introduced by Ramon in 1923 for the immunization of man. Diphtheria toxoid is produced by treating standardized toxin with 0.3 per cent formalin at 37°C for approximately one month. Toxoid which has been tested for safety and immunizing capacity is injected subcutaneously in three doses of 0.5, 1.0 and 1.0 ml. at one to three weekly intervals. Immunity is manifest by production of a negative Schick reaction in approximately 95 per cent of recipients of toxoid in four weeks after injection.

**Alum-precipitated toxoid,** which consists of toxoid rendered insoluble by precipitation with potassium alum (potassium aluminum sulfate), appears to have some advantage in that the insoluble toxoid is more slowly absorbed and provides a more prolonged antigenic stimulus. At the present time alum-precipitated toxoid is usually administered in two or three injections of 1.0 ml. at three to four weekly intervals. The single injection method (one injection of alum-precipitated toxoid) is no longer recommended because of failure to elicit adequate immunity in a high proportion of persons.

Toxoid immunization is followed by few untoward reactions in young children. A proportion of adolescent and adult persons, however, exhibits an unusual sensitivity to injection of either toxoid or alum-precipitated toxoid and reacts unfavorably to these materials. Sensitivity may be detected prior to immunization by means of the Moloney intradermal test with toxoid. Persons who react unfavorably may be immunized with toxoid-antitoxin or toxin-antitoxin mixtures.

**Control.** The control of diphtheria may be considered from the point of view of long-term prevention of the disease and the control of existent epidemics. It has been indicated that *C. diphtheriae* is widely distributed throughout the world and is firmly entrenched in a carrier population. Furthermore, this infected group is constantly changing through transference of the bacteria. It is impractical, therefore, from the public health point of view to attempt to eradicate the microorganism. On the other hand, detection of carriers among contacts of patients is often practical and should be attempted in the case of localized epidemics, hospital personnel, etc. Long-term prevention, however, is most entirely dependent upon immunization procedures to create an immune population with a density of susceptibles too low to support the disease (see Chapter 43). In practice this means extensive and continued programs of immunization of infants, preschool and school age children.

In the control of epidemics, the presence of diphtheria demands the detection of cases by temperature records and particularly physical examinations, and the

isolation and immediate treatment of persons ill with the disease. Exposed persons, such as school and familial contacts, should be closely supervised in order to detect early diphtheria. Further investigation should include culture of the nose and throat for *C. diphtheriae*, followed by isolation and appropriate treatment of infected persons, both susceptible and immune carriers. The use of antitoxin have already been described. Infected individuals are commonly required to be isolated until two consecutive daily cultures fail to reveal *diphtheriae*. Tonsillectomy has been found of some value in the cure of chronic pharyngeal carriers. In addition, the Schick test may be performed on contacts and susceptible persons may be actively immunized with toxoid in order to prevent a repetition of the epidemic. However, in children the high percentage of positive reactors makes it practical to immunize all of the exposed group without using the Schick test before immunization.

The disease diphtheria, which is essentially an intoxication, is not relieved by penicillin therapy, and the use of the antibiotic is of questionable value in the cure of carriers of the infection. It should be remembered that the carrier state, whether convalescent or chronic, tends toward natural cure over a period of time without any treatment.



## THE MYCOBACTERIA— TUBERCULOSIS AND LEPROSY

The Mycobacteria are slender rods which are acid-fast, that is, they are stained with difficulty but once staining has occurred they are not decolorized by acidified decolorizing agents. The genus includes a number of parasites of man and the lower animals, including birds, mammals, reptiles, frogs and fish, as well as species which are saprophytic in the soil. The most important members of the genus are *Mycobacterium tuberculosis* (the tubercle bacillus), proved by Robert Koch (1882) to be the cause of human tuberculosis, and *Mycobacterium leprosy*, described by Hansen (1874) in the tissues of human leprosy. The Mycobacteria, like the Corynebacteria, are classified in the family Actinomycetaceae because of the production of filamentous and irregular or branched forms. These organisms are gram-positive, nonmotile, aerobic and nonsporulating.

### MYCOBACTERIUM TUBERCULOSIS TUBERCULOSIS

Evidence from medical history and the study of ancient skeletons indicate that tuberculosis in its many forms, including the pulmonary disease (known as consumption or phthisis) and abscesses of the bones and soft tissues, has been a plague of man since antiquity. The unity of these various forms of tuberculosis was recognized by Laennec, and the epidemiology, established by Villemin (1848), was rapidly confirmed following Koch's isolation of the tubercle bacillus in 1882.

**Morphology.** The tubercle bacilli are slender straight or curved rods, 0.5 to 1.0  $\mu$  in length, although occasionally granular or filamentous and branched forms may be seen. The individual organisms are arranged singly, in pairs or in long chains. The bacteria are stained with difficulty by the usual bacteriological stains, but are readily dyed by the Ziehl-Neelsen technique, which consists of staining heat-fixed preparations for 2 to 5 minutes with carbol fuchsin (basic fuchsin 1 per cent, alcohol 10 per cent, phenol 5 per cent in water), decolorizing with dilute acid (2 per cent hydrochloric acid in 70 per cent alcohol) and counterstaining with a contrasting dye, such as methylene blue. The bacilli retain the primary dye, whereas the background of debris, body cells, pus or sputum is blue. The retention of dye during decolorization with acid appears to be related to the

presence of wax in the cells. Alternative methods of staining include staining overnight in carbol fuchsin at room temperature or staining with fluorescent carbol-auramine. The latter preparations are examined in ultraviolet light. Although tubercle bacilli are typically acid-fast, nonacid-fast granules and bacteria have been described by Much in tuberculous abscesses in the absence of demonstrable typical organisms. The nature of the Much granules remains undecided although they have been thought to represent either a part of a life cycle of the tubercle bacillus or debris and degradation products. Tubercle bacilli are gram positive, although heat must be used to stain the cells.

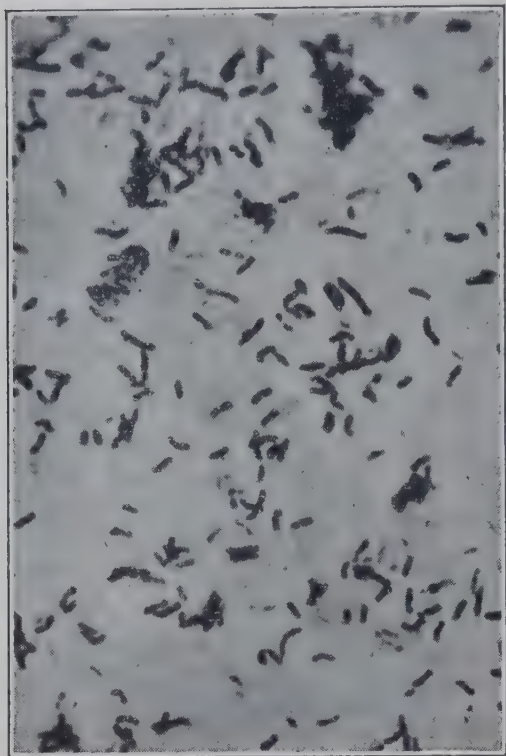


Fig. 156. Photomicrograph of stained smear of human tubercle bacilli. (Magnification approximately  $\times 1,600$ .) (Kral.)

the tubercle bacillus or debris and degradation products. Tubercle bacilli are gram positive, although heat must be used to stain the cells.

On suitable culture media, such as glycerol agar or coagulated serum and other media, the growth of human tubercle bacilli is nodular or wrinkled, very tenacious and gray to orange-yellow in color. On liquid media the growth forms a wrinkled pellicle although a uniform turbidity is obtained in Dubos' medium. The avian variety produces yellow or pink growth, whereas the bovine tubercle bacilli are not pigmented.

**Variation.** Smooth and rough forms of tubercle bacilli have frequently been described, although the permanence of the forms and their relationship to virulence is not clear. In many instances smooth and rough growth appears to be related to the type of medium on which the bacteria are grown rather than to true variance. In smears from smooth cultures the bacteria are typically arranged in regular packets

whereas those from rough stains are grouped in irregular masses. The differences in arrangement are related to the postfissional movement of the growing organisms.

Loss of virulence of a strain of bovine tubercle bacillus has, however, been produced by prolonged cultivation of the organism on a medium containing bile, glycerol and potato. The resulting culture, known as Bacille Calmette-Guérin (B.C.G.) after the French investigators, appears to be permanently avirulent and has been used for preventive vaccination against human tuberculosis. The loss of virulence is unrelated to cultural changes.

**Physiology.** Tubercle bacilli have a restricted temperature range of growth; the human and bovine varieties grow best at  $37^{\circ}\text{C}$ , the avian type at  $40^{\circ}\text{C}$ . None develop below  $30^{\circ}\text{C}$  or above  $42^{\circ}\text{C}$ . Cultures require oxygen and are benefited by  $\text{CO}_2$ . The bacteria, particularly freshly isolated strains, grow slowly



several weeks usually being required for the appearance of visible growth. Development occurs at either a slightly acid or alkaline  $pH$ . Acid-fast bacteria in general are not more resistant to heat than are other sporulating bacteria, a fact of great importance, since they are destroyed by sterilization and by usual methods of heat sterilization. They are, however, usually resistant to chemical disinfectants, to acids and alkalis and to drying. Such chemical disinfection is effective only after prolonged exposure of the tubercle bacilli. Furthermore, virulent organisms may survive prolonged drying in sputum and dust if protected from sunlight. They are rapidly destroyed by direct exposure to ultraviolet light.

The biochemical reactions are poorly known. Glucose appears to be uniformly utilized and arabinose and sucrose are attacked by some strains. Acid is not produced, either because of complete oxidation to  $CO_2$  and water or because of simultaneous production of ammonia. The organisms produce  $H_2S$  but not indole, and growth in milk results in no visible change. The biochemical reactions are of not of practical value in differentiation of the tubercle bacilli.

Tubercle bacilli require complex media for growth, particularly for primary isolation from infectious material. Generally media containing egg or potato and glycerol, such as Petroff's, Löwenstein's or Herrold's media, all of which contain glycerol, and sterile egg or egg yolk are used. Glycerol and, to a lesser degree, potato are stimulatory to the growth of the tubercle bacillus, and necessary growth factors are presumably supplied by the sterile egg and potato. Bacteriostatic dyes, such as congo red and malachite green (Löwenstein) or gentian violet (Petroff) are frequently added to inhibit growth of contaminating bacteria. Laboratory cultures of *Myco. tuberculosis* may be cultivated in synthetic media, such as that of Long or Sauton, both of which contain inorganic salts, phosphate ions, asparagin and glycerol. The latter medium is used in the production of B.C.G. vaccine. Excellent growth of both initial and stock cultures of tubercle bacilli is obtained in Dubos' medium (enzymatic casein-hydrolysate, asparagin, Tween 80 [esters of oleic acid], inorganic salts and bovine serum albumin). Furthermore, dispersed growth may be obtained in liquid medium because of the surface wetting effect of the oleic acid esters.

The vitamin requirements of tubercle bacilli are incompletely known. Growth is stimulated by vegetable juices, fractions of serum albumin and extracts of heated hen's eggs and potato. The stimulatory activity is alcohol soluble, is not replaced by a variety of known bacterial growth factors, and remains unidentified. Yeast extract appears to increase growth in some, but not in all, media.

**Chemistry of the Tubercle Bacillus.** The chemistry of the tubercle bacillus is particularly interesting because certain of the carbohydrates, lipids and proteins have been found important to the development of lesions typical of tuberculosis and to immunity. The nucleic acid of *Myco. tuberculosis*, known as tuberculinic acid, is somewhat unusual in that it contains thymine, a substance typical of animal nucleic acids. The lipoidal components are of particular interest in that these materials have been found active in the stimulation of

tubercle-like lesions and appear to be responsible for the acid-fast property of tubercle bacillus. The lipoids and carbohydrates include neutral fats, phospholipids and waxes. The waxes, which are complex compounds of carbohydrates and fatty acids, contain a characteristic acid known as **mycolic acid**, and are acid-fast. Two other fatty acids, **tuberculostearic acid** and **phthioic acid** are peculiar to the tubercle bacillus. Phthioic acid is of importance because it has the property of stimulating tubercle-like lesions containing typical epithelioid cells and giant cells in experimental animals.

**Tuberculin.** Koch first prepared tuberculin in the form of filtrates of cultures of tubercle bacilli. Although tuberculin has since been prepared in numerous ways and has been partially purified, all preparations cause a specific local or generalized hypersensitive reaction in infected animals and man, which is of practical importance in the diagnosis of human and bovine tuberculosis. The preparations of tuberculin are commonly used at the present time. The first of these is Koch's **old tuberculin** or **original tuberculin** (O.T.) prepared by heat concentrating old glycerin broth cultures of tubercle bacilli to one-tenth the original volume and removing the bacteria by filtration. Old tuberculin is thus a crude preparation of the bacterial culture containing the heat-resistant soluble cell substances. In recent years Seibert and Long have purified the active material of tuberculin from cultures in synthetic medium by trichloroacetic acid precipitation and have found it to be tuberculo-protein. The purified active material is generally referred to as **Purified-Protein-Derivative** (P.P.D.). Tuberculin prepared from either human or bovine tubercle bacilli is active, but that from avian bacilli is unsatisfactory. Immunization with tuberculin is unsatisfactory.

**Types of Tubercle Bacilli.** Three varieties or types of tubercle bacilli are commonly recognized, the human, the bovine and the avian. The varieties of tubercle bacilli may be differentiated by cultural tests and by pathogenicity for experimental animals. The human variety grows luxuriantly on suitable media at 37° C, produces pigment, is stimulated by glycerol and is pathogenic for the guinea pig but not for the rabbit or chicken. The bovine type, on the other hand, grows less luxuriantly, is not stimulated by glycerol in an adequate medium, does not produce a pigment and is pathogenic for both the guinea pig and rabbit but not for the chicken. The avian bacilli grow best at 40° C or above, are pigmented, are stimulated by glycerol and are more virulent for the chicken than the guinea pig or rabbit.

**Human Tuberculosis.** Human tuberculosis may affect any tissue of the body and may be caused by either the human or bovine varieties of tubercle bacilli. Some tissues are, however, more frequently infected than are others. The organisms most commonly find entry in the respiratory or gastro-intestinal tracts, less frequently invade the conjunctiva and the skin and rarely the genito-urinary tract. Tuberculosis of the respiratory tract (pulmonary tuberculosis, consumption, or phthisis) is at the present time responsible for over 90 per cent of fatal human infections, although bone and joint tuberculosis, infection of the lymph



s (tuberculous lymphadenitis or scrofula), soft tissue abscesses, gastrointestinal and kidney infection, as well as generalized (miliary) tuberculosis and tuberculous meningitis are also frequent.

Irrespective of the site of infection, the basic lesion of tuberculosis is the tubercle, which is a small visible or microscopic nodule produced by the body in reaction to the tubercle bacillus and its products. The tubercle has a characteristic appearance grossly and microscopically. In response to the invading bacilli the surrounding defense cells produce typical, elongated epithelioid cells, the large multinuclear giant cells, which form the center of the tubercle. At the periphery there is an accumulation of defense cells, particularly lymphocytes and other mononuclear cells, although leucocytes are present. The young individual tubercle is a small, firm, white nodule. As the lesion enlarges, necrosis occurs in the center of the tubercle, giving rise to caseation, that is, production of cheese-like pus. A tuberculous lesion may extend by confluence of tubercles to produce large abscesses or, with drainage of the pus, abscess cavities. Healing is manifest in this, as in other infections, by production of fibrous tissue and walling-off of the infected area and eventually by scar formation. In tuberculosis the healing process is often accompanied by calcification or even ossification of the lesion, producing a permanent, indurated, hard nodule. At times, acute

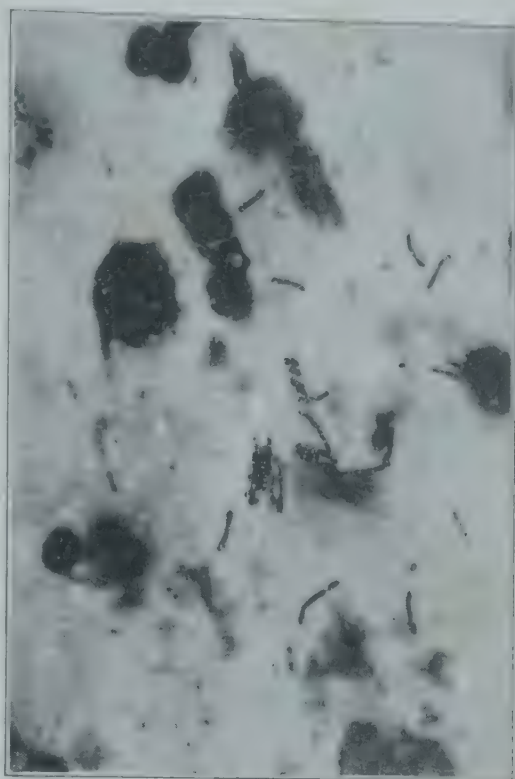


Fig. 157. Tubercle bacilli in sputum. Photomicrograph of stained smear. (Magnification  $\times 1,600$ .) (Kral.)

inflammation (the exudative reaction) is encountered in tuberculosis, particularly in highly allergic individuals or in the presence of overwhelming infection. The response of the body to tuberculosis is a particular form of chronic inflammation, called a **granuloma** which, although typical of tuberculosis, also occurs in a number of other chronic infections. The early lesions of tuberculosis appear to be stimulated directly by the tubercle bacillus and its products, such as mycolic acid, whereas later, interference with blood supply, toxicity of bacterial products, hypersensitivity and the processes of repair contribute to the development of the disease.

Spread of the tuberculous process from the initial site of infection to other parts of the body may occur by several routes. First, the infection may spread by continuity to adjacent tissues, in which case the infection tends to follow the natural divisions. In this way the pleura and pericardium may become infected. In pulmonary tuberculosis, communication of a tuberculous cavity with the air

passages results in the presence of tubercle bacilli in the bronchial secretions the sputum. The organisms may thus be spread from one lesion in the lung to other areas through the air passages. Tubercle bacilli are frequently spread through the lymphatic system and directly or indirectly by the blood. Tuberculous infection of the regional lymph nodes (tuberculous adenitis) which receive the lymphatic drainage from the initial site of invasion is frequently observed

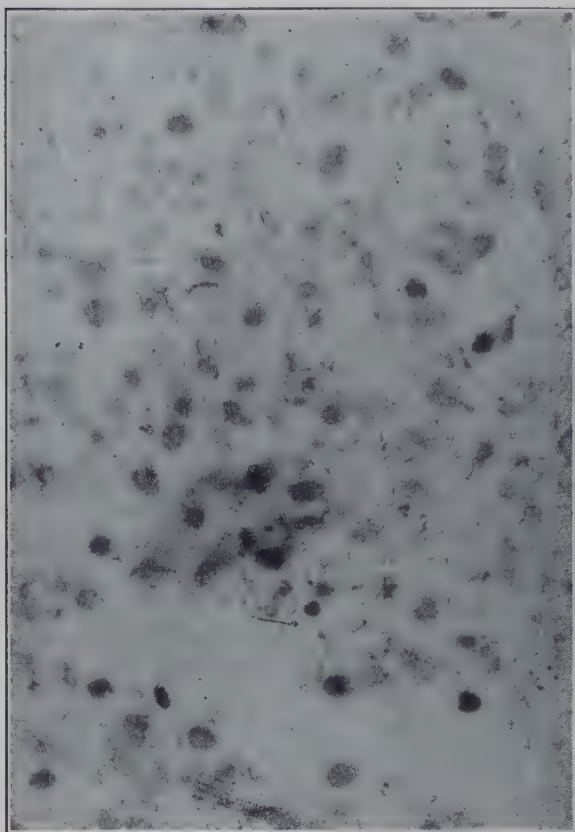


Fig. 158. Photomicrograph of a section of a tuberculous lesion showing exceptionally large numbers of tubercle bacilli. (Magnification approximately  $\times 1,400$ .) (Courtesy of Dr. George Gomori.)

in children, less often in adults. Lymph nodes most frequently involved are those in the neck (cervical lymphadenitis) probably by invasion in the pharyngeal region, the abdomen (mesenteric lymphadenitis) secondary to intestinal tuberculosis and the hilar region of the lungs (mediastinal lymphadenitis) secondary to pulmonary infection. Tuberculous infection of the meninges (meningitis), bones and joints, the kidneys, glands of internal secretion, particularly the adrenal gland, the reproductive organs, other deep tissues and generalized miliary tuberculosis result almost entirely from spread by the blood from primary lesions elsewhere.

Tuberculosis differs considerably in children and other previously unexposed individuals from that usually seen in adults. The childhood type of tuberculosis is characterized by a primary lesion, usually in the lung, and infection of the regional lymphatics. Although there is a tendency for rapid

spread, development of resistance is usually accompanied by arrest and eventually healing of the process with calcification, giving rise to the healed primary complex or Ghon tubercle.

The disease may progress, however, in which case there is rapid multiplication of the bacteria and extension or generalization of the infection from necrotic poorly localized lesions. Miliary tuberculosis and meningitis are more often encountered in children, as are lesions of the lymph nodes, bones, spleen, kidneys and other deep tissues. The adult type of tuberculosis, on the other hand, appears to result from reinfection of the hypersensitive, partially resistant individual and is a slowly progressing disease which tends to localization and fibrous healing of the lesions. Caseation necrosis occurs in moderately advanced tuberculosis and, of epidemiological importance, frequently is subject to cavitation.



munication with a bronchus. In such instances, tubercle bacilli are present in the sputum and the individual is highly infectious.

The human type of the tubercle bacillus is the most frequent cause of human infection. This organism, which in nature is almost exclusively a parasite of man, is spread from person to person, and most often invades the respiratory tract. Bovine tubercle bacilli, on the other hand, are natural pathogens of cattle and are acquired by man through contact with infected cattle or more commonly through ingestion of contaminated milk and other food. Bovine type bacilli, therefore, more often invade through the intestinal tract and produce infection in lymph nodes. Because of existent control measures, bovine infection is at the present time relatively infrequent in the United States.

**Epidemiology** (see also Chapter 44). Tuberculous infection is widely distributed throughout the world, but it is particularly frequent in urban areas and in regions where crowded living conditions with poor sanitation prevail. The prevalence of bovine infection is directly related to the degree of infection in cattle and to the use of raw milk. As a result of preventive and therapeutic measures the mortality from tuberculosis has declined remarkably and specifically during the present century (see Chapter 44). The prevalence of infection, however, has not declined proportionately. The proportion of individuals who show evidence of tuberculous infection increases progressively from birth throughout life, so that 50 to over 90 per cent of adults examined at autopsy show evidence of infection. The percentage of persons who react positively to the tuberculin test also increases progressively with increasing age, approximately 75 per cent of adults giving a positive reaction. It is clear that tuberculous infection is widely disseminated. The amount of medically important disease is, however, less extensive. Surveys in many groups of the population have indicated an incidence variable between 3 per 1000 and 4 per 1000 population.

Morbidity and mortality statistics, as well as experimental studies, indicate a number of factors predisposing or contributing to development of active tuberculosis. The degree of exposure and the development of disease are increased by familial exposure to an infectious patient; children, adolescents and young adults are particularly affected. The disease is more frequent and more severe among Negroes and other nonwhite groups than among the white population, a fact related to degree of exposure and socio-economic factors and possibly to underlying factors of racial susceptibility. Predisposing socio-economic factors include insufficient or inadequate nutrition, overcrowded living conditions, physiological stress and occupational hazard. The existence of genetic susceptibility and resistance to tuberculosis in man is problematical, although it is suggested by the familial and racial incidence of infection. However, the degree of exposure within infected groups is increased over that of the general population. The disease is not hereditary, and congenital transmission is rare. Furthermore, programs for the prevention of tuberculosis in children of tuberculous families may be highly successful.

Infection with human type bacilli is transmitted chiefly by droplet and air-

borne infection. The resistance of tubercle bacilli to drying and, hence, survival in dried sputum and settled dust particles, as well as the contamination of animate articles, contribute to dissemination. As has been indicated, bovine tuberculosis is acquired from infected cattle.

**Pathogenicity for Animals.** Tuberculosis naturally affects a number of domestic animals, particularly when these animals are confined in small enclosures. Bovine tubercle bacilli are highly virulent for cattle, horses, pigs and other domestic mammals, often producing a highly fatal, generalized tuberculosis. Human type bacilli, on the other hand, give rise only to localized infections. Avian tubercle bacilli are infectious for a few mammals, but are primary pathogens of birds, the domestic chicken being frequently infected. Tuberculosis is commonly observed in wild animals in captivity, but is presumably rare under natural conditions.

A number of domestic, wild and laboratory animals may be infected experimentally, although guinea pigs, rabbits and chickens are most commonly used for experimental purposes. The lesions are essentially the same as those described under human tuberculosis. The location of the primary site is, of course, determined by the route of inoculation, and the extent of infection varies with the type and quantity of bacilli. In the guinea pig, inoculated subcutaneously in the groin with human type tubercle bacilli, a local abscess develops, followed by extension to the inguinal lymph nodes and frequently miliary tuberculosis with tubercles in the liver, spleen and other tissues. Three to six weeks are usually required for full development of the disease. The pathogenicity for laboratory animals of the different types of tubercle bacilli has been discussed previously.

**Diagnosis of Tuberculosis.** The demonstration of tubercle bacilli in the bodily discharges, fluids and tissues of tuberculous individuals constitutes a unequivocal diagnosis of the disease. Tubercle bacilli are commonly found in the pus of tuberculous abscesses, in the cerebrospinal fluid in tuberculous meningitis, in the urine in tuberculosis of the genito-urinary system, in the sputum or gastric washings in pulmonary tuberculosis with cavitation, and in the feces in intestinal tuberculosis. However, they may not be demonstrated in the latter instances when the lesions do not communicate with the air spaces or the lumen of the genito-urinary or intestinal tract.

The examination of material for bacilli may be made by microscopic, cultural and animal inoculation tests. In the microscopic test smears of pus, sputum, cerebrospinal fluid or other materials, or sections of tissues are stained by the acid-fast technique and are examined under the microscope for the presence of bacilli. The number of organisms is highly variable, so that their demonstration may require concentration methods. The bacilli in sputum may be concentrated by first digesting the other constituents of sputum and then concentrating the bacilli by centrifugation. The digestion is carried out by treating the sputum for 30 minutes at 37° C with (1) equal parts of 50 per cent antiformin (a proprietary alkaline hypochlorite solution) or preferably (2) equal parts of 4 per cent sodium



oxide; treatment with antiformin or alkali is destructive to other bacteria. A rough estimate of the number of bacilli is obtained by the Gaffky microscopic count of an unconcentrated specimen; the specimen is graded from Gaffky No. 1 to 10 according to the number of organisms seen. Gaffky No. 1 and No. 2 have occasional bacilli, Gaffky Nos. 3 to 7 average from 1 to 25 bacilli in each microscopic field and Nos. 8 to 10 have 50 or more organisms per microscopic field. Concentration, either by centrifugation or chemical precipitation, is required for the demonstration of tubercle bacilli in urine. Furthermore, the virulent, acid-fast *Mycobacterium smegmatis*, which is commonly present in the normal genital secretions, may be present in urine. The tubercle bacillus may be differentiated from *Myco. smegmatis* by virulence tests.

Cultural methods for the demonstration of tubercle bacilli have until recently provided little information not obtained by microscopic examination or virulence tests. However, the rapid cultural method developed by Dubos may prove a valuable confirmatory procedure.

Animal inoculation tests are usually carried out in guinea pigs. Animals inoculated subcutaneously in the groin with material freed of other bacteria are observed weekly for weight loss and development of typical lesions. Animals are sacrificed upon the appearance of lesions or after eight weeks and are examined at autopsy for the presence of tuberculosis at the site of inoculation, in inguinal lymph nodes and in the liver and spleen. Smears of lesions should be examined for acid-fast organisms.

**The Tuberculin Reaction.** A positive reaction to tuberculin is indicative of hypersensitivity and hence infection with tubercle bacilli. In man and animals demonstration of hypersensitivity has diagnostic value. In man the tuberculin test is a skin test, performed with either old tuberculin (O.T.) or Purified Protein Derivative (P.P.D.). The percutaneous scratch test of von Pirquet, the intradermal Mantoux test or the Vollmer patch test may be used. The test most frequently used is the Mantoux test in which either 0.1 ml. of a 1:1000 or 1:10,000 dilution of standardized O.T. (0.1 or 0.01 mg.) or 0.00002 mg. P.P.D. is injected intradermally. Larger amounts may be cautiously used in the event of a negative reaction to the first injection. In a positive reaction redness and swelling (induration) develop at the site of injection within 48 hours. In individuals having active tuberculosis an inflammatory response also occurs about tuberculous lesions (perifocal reaction) and a systemic response with fever may develop. Since such reactions are undesirable, tuberculin tests should not be performed in patients with active tuberculosis. In cattle both a local edematous reaction and the febrile response have diagnostic significance. The test is usually performed by injecting tuberculin into the caudal skin fold.

Interpretation of the tuberculin reaction presents some difficulty. In cattle a positive reaction is generally interpreted to mean active infection, particularly when an animal previously negative becomes positive, and suitable control measures are invoked. In man, hypersensitivity to tuberculin should not necessarily be interpreted to mean active disease, since previously healed or clinically

unimportant infection results in hypersensitivity of long duration. A positive reaction to tuberculin indicates some infection, past or present, and should be followed by additional diagnostic examination. The test finds its greatest value in the examination of children for tuberculosis. In the young age groups, tuberculin activity is normally low and sensitizing infection is generally recent, so that a positive tuberculin reaction, particularly when preceding tests have been negative, suggests active infection. The actual diagnosis must, however, be established by other clinical and laboratory methods. The test is of value in epidemiological surveys and in the differentiation of tuberculous lesions from those of histoplasmosis and coccidioidomycosis.

At the present time x-ray examination is the most reliable method for detecting tuberculosis, and it is positive in a high proportion of cases in which bacteriological examination is negative. Furthermore, the x-ray examination must be relied upon to determine whether infection is active, requiring treatment and public health control, or inactive and medically unimportant.

**Immunity in Tuberculosis.** Resistance to tuberculous infection is shown by the frequency with which small infections are healed without development of clinical tuberculosis and by the reinfection adult type of tuberculosis. Although antibodies, such as agglutinins, precipitins and complement-fixing antibodies, may be demonstrated in low titer, the characteristic immune response of the animal body is the development of hypersensitivity to tubercle bacilli and their products. Thus, in both man and animals a second infection with tubercle bacilli is followed by a rapidly developing inflammatory reaction, with necrosis and a marked tendency toward fibrous scarring and healing of the infection. Subcutaneous inoculation of tubercle bacilli into a tuberculous guinea pig results in a necrotic ulcer which heals within a few days, whereas in a normal guinea pig a progressive infection develops in the course of several weeks. The rapid reaction in the tuberculous guinea pig, known as **Koch's phenomenon**, represents hypersensitivity and, to a degree, immunity. The same reaction is produced if the second injection consists of killed tubercle bacilli or tuberculin. Larger injections result in untoward general reactions, and even death of the animal. There is, therefore, resistance to reinfection, which is related to hypersensitivity to the tubercle bacillus. This resistance is, however, insufficient to prevent infection with relatively larger doses of tubercle bacilli.

**Active Immunity.** The idea that hypersensitivity is an evidence of resistance to infection in tuberculosis forms the basis of many attempts to produce effective immunity by vaccination. Tuberculin itself and killed tubercle bacilli were early found to induce inadequate protection. Vaccination with *Bacillus Calmette-Guérin* (B.C.G.) was given extensive trial soon after development of this attenuated strain. Vaccination is performed with living bacteria in a manner analogous to vaccination against smallpox, and is followed by development of one or more small cutaneous tubercles at the site of inoculation. These heal within a few weeks and result in hypersensitivity to tuberculin in a high proportion of vaccinated persons. Vaccination with B.C.G. has been given extensively



in France and in the United States, especially among children. The accumulated evidence suggests a reduction in the number of cases of clinical tuberculosis in vaccinated as compared with control groups. The evidence of protection is, however, as yet insufficiently good and the practical value insufficiently determined to justify large-scale vaccination of the human population. Vaccination with living bacilli is not without some danger of undesirable reaction.

**Chemotherapy.** Recently *Mycobacterium tuberculosis* has been found susceptible to certain chemotherapeutic agents, among them streptomycin, promin, promizole, para-amino salicylic acid. Streptomycin is now being used in the treatment of human and experimental tuberculosis. Indications are that this substance will have value in the treatment of early pulmonary, meningeal and miliary tuberculosis but is ineffective in active, long-standing pulmonary tuberculosis.

## MYCOBACTERIUM LEPRAE

### Leprosy

Leprosy has been known since antiquity and, although relatively infrequent at the present time, it is by no means an extinct disease. Records indicate that leprosy was widespread in the western world during the Middle Ages, and that the disease has declined to a relatively low incidence. It remains prevalent in the tropics, however, including the islands as well as the major continents. In the United States leprosy is present in a number of foci in the South and is occasionally encountered elsewhere.

Leprosy occurs in two clinical forms, the cutaneous or nodular and the anesthetic or neural. In the former, nodular lesions resembling tubercles and other massive granulomatous lesions called lepromata are present in the skin and deep tissues. Neural leprosy affects primarily the nervous tissues. Leprosy is ordinarily an extremely chronic disease of many years' duration, which may be disfiguring because of the lepromata and mutilating in the loss of fingers, toes and other members. The disease is not often fatal. The disease process is not continuously progressive, but in the course of years it may show improvement, may demonstrate periods of quiescence and acute spread.

**Bacteriology.** *Mycobacterium leprae* (Hansen's bacillus) may be found in large numbers in the lesions of leprosy and at times in the sputum or nasal discharges of lepers. The bacillus is acid-fast and morphologically indistinguishable from *Mycobacterium tuberculosis*. The organism is included with the *Mycobacteria* because of its resemblance to the tubercle bacilli. In the tissues it is observed in packets of parallel cells within large phagocytic cells called lepra cells. The organisms are found in all cases of leprosy, and the bacteriological diagnosis is made by demonstration of typical organisms in smears, sections of tissues or in sputum. *Mycobacterium leprae* has not been cultivated with certainty, although acid-fast bacilli have been isolated from leprosy lesions. The disease has not been reproduced in animals by inoculation of either cultures or macerated

leprous tissues. Although leprosy is generally conceded to be a bacterial disease caused by *Myco. leprae*, it is clear that only the first of Koch's postulates, that the constant association of the bacterium with the disease, has been fulfilled.

The method of transmission and the route of infection are likewise poorly understood. The infectious nature of the disease is illustrated by development of leprosy in persons having personal contact with lepers, a few positive attempts to transmit the disease to man and by the decline of leprosy following segregation of lepers. The disease is presumably contracted by close personal contact, often familial contact during childhood. It is most prevalent among lower socioeconomic groups living in crowded and unhygienic conditions. Nodular leprosy is the more infectious form, and organisms may be present in nasal secretions and sputum. The incubation period presumably may extend over many years.

The infectivity of leprosy is very low, probably because of resistance to infection, and infection is associated with hypersensitivity to extracts from leprous nodules (the **lepromin reaction**) and to a number of other acid-fast bacteria.

### OTHER MYCOBACTERIA

**Rat Leprosy.** A transmissible disease which somewhat resembles human leprosy has been described in naturally infected rats from many parts of the world. Rats are resistant to inoculation with material from human leprosy, so that a relationship, if any, between human and murine disease has not been established. The murine organism is called *Myco. leprae murium*.

**Johne's Disease.** Cattle and to a lesser extent sheep are subject to Johne's disease, a highly fatal specific enteritis caused by *Myco. paratuberculosis*. The organisms, which resemble somewhat avian tubercle bacilli, are present in large numbers within the lesions. Indeed, infected animals may react to avian tuberculin as well as to extracts of *Myco. paratuberculosis*, but not to bovine tuberculin. The disease is widespread.



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### THE GRAM-NEGATIVE ENTERIC BACTERIA

The enteric bacteria are a large group of nonsporulating, gram-negative bacteria which are of medical interest as normal parasites and important pathogens of man and animals. Many other members of the group are widely distributed saprophytes of the soil and pathogens of plants which will not be considered here. Enteric bacteria are customarily divided into subgroups, the cultural reactions of which are summarized in the accompanying table. The coliform, *aerogenes* and Friedländer groups are closely related, lactose-fermenting bacteria. The *Salmonella* group characteristically does not ferment lactose and sucrose, does not produce indole or pigment or liquefy gelatin. The dysentery group is comprised of nonmotile, dextrose-fermenting bacteria. Members of the *Proteus* group ferment dextrose and sucrose, peptonize milk and liquefy gelatin. *Pseudomonas* is a group of pigmented bacteria closely related to saprophytic bacteria and plant pathogens and not ordinarily included with the enteric bacteria. However, the parasitic species, *Pseudomonas pyocyanea*, is sometimes found in the intestine and must be distinguished from the enteric bacteria. It is included as a matter of convenience. The spiral bacteria, discussed in the succeeding chapter, are likewise related to the enteric bacteria and are of medical importance as intestinal pathogens.

The enteric bacteria as a group are well defined and distinctive, and each subgroup is as a whole comprised of bacteria with common physiological and immunological characteristics. The groups are, however, similar to each other, so that if all types and species of bacteria are considered they form an interesting series of microorganisms from the *coli-aerogenes* group and associated groups to the *Proteus*, *Salmonella* and dysentery groups. The subgroups, as well as species and types, none the less have value in the identification of pathogens in the classification of these microorganisms.

#### THE COLI-AEROGENES GROUP

The lactose-fermenting enteric bacteria may be divided into the *coli-aerogenes* group, described by Escherich (1886) and the related Friedländer group of nonsporulating bacteria. *Bacterium coli* (*Escherichia coli*, *Bacillus coli*) is a strict

TABLE 12. CULTURAL REACTIONS IMPORTANT IN RECOGNITION OF GROUPS OF THE ENTERIC BACTERIA

BACTERIAL GROUP	MOTILITY	FERMENTATION			METHYL RED TEST	VOGES-PROSKAUER REACTION	ELJHKMAN TEST	GROWTH IN CITRATE MEDIUM	RUSSELL'S MEDIUM		INDOLE	PRODUCTION OF H <sub>2</sub> S	LITMUS MILK	LIQUEFACTION OF GELATIN	PRODUCTION OF PIGMENT
		DEXTROSE	LACTOSE	SUCROSE					BUTT	SLANT					
Coliform	+	G	G	G	+	-	+	-	G	A	+	-	AC	-	-
<i>Aerogenes</i>	±	G	G	G	-	+	-	+	G	A	-	-	AC	-	-
Friedländer's	-	G	G	G	+	-	-	+	G	A	-	-	AC	-	-
<i>Salmonella</i>	±	GA	-	-		-			GA	-	-	+	AK	-	-
Dysentery	-	A	±	±					A	-	±	-	AK	-	-
<i>Proteus</i>	+	G	-	G							±	+	KP	±	-
<i>Pseudomonas</i>	+	A	-	-							-	+	P	+	-
<i>Alcaligenes faecalis</i>	+	-	-	-					-	-	-	+	K	-	-

\* +, positive; G, gas; A, acid; C, coagulation; K, alkaline; P, peptonization; ±, variable (positive or negative); -, no change or negative; no entry, unimportant or unknown.



site of the intestinal tract of man and the higher animals. *Bacterium aerogenes* (*Aerobacter aerogenes*, *Bacterium lactis-aerogenes*), on the other hand, is a common saprophyte found in soil, water and milk. Although typical *Bact. coli* and *Bact. aerogenes* are easily recognized and may be separated from other groups of enteric bacteria, intermediate coliform organisms possessing properties of both are now clearly recognized. Furthermore, the *coli-aerogenes* group is antigenically and culturally related to the *Salmonella* group of enteric bacteria.

**Bacterium Coli (Escherichia Coli).** *Bacterium coli* is characteristically found in the intestinal contents and feces of healthy man and other vertebrates and is particularly numerous in the lower colon. Although this bacterium is a normal parasite, it may at times be pathogenic, giving rise to serious or fatal



Fig. 150. (Left) Colonies on blood agar and (right) photomicrograph of *Bacterium coli*. (Magnification approximately  $\times 1,400$ .)

infections in many locations in the body. The predominance of *Bact. coli* in the intestinal contents, its absence from unpolluted soil and water, the ease of its identification and the similarity of its resistance to deleterious conditions to that of the enteric pathogens have recommended use of this bacterium as an index of fecal pollution. Its recognition is, then, of great practical importance.

**Morphology.** *Bacterium coli* is typically a motile, nonsporulating, gram-negative bacterium which varies in size from short coccobacilli to long slender rods. Usually the rods measure 0.5 by 1.0 to 4.0  $\mu$  and may be arranged singly, in pairs and chains. Motile strains possess peritrichous flagella.

Colonies on ordinary media are several millimeters in diameter, grayish-white, opaque, and smooth with slightly irregular margins. In broth an even turbidity develops and a heavy sediment is produced.

**Metabolism.** *Bacterium coli* grows well either aerobically or anaerobically at room or body temperatures and, of differential value, develops at 46° C. (The standard test for fermentation of glucose at 46° C is positive.) The organism is killed by heating at 60° C for 30 minutes and is not unusually resistant to drying and disinfectants. Growth is abundant on all ordinary laboratory media and in synthetic media containing ammonium salts and glucose, without added vitamins. In contrast to *Bact. aerogenes*, *Bact. coli* is unable to grow in a synthetic medium lacking citrate ions as the carbon source.

Fermentation reactions have differential value. Acid and gas ( $\text{CO}_2$  and  $\text{H}_2$ ) are produced in dextrose, levulose, galactose, lactose, maltose, arabinose, xylose, rhamnose and mannitol. Some strains ferment sucrose and glycerol but cannot ferment carbohydrates such as starch and glycogen are not attacked. Fermentation is usually rapid, although in some instances acid and gas production in lactose broth is delayed. An acid coagulation is produced in milk; gelatin is not liquefied; indole and nitrites are produced. The **methyl-red test** for acidity in glucose broth is positive after 3 to 4 days' incubation at  $30^\circ \text{C}$ , indicating a high acid production; the **Voges-Proskauer reaction** for acetyl-methyl-carbinol in glucose in glucose-peptone medium is negative. In the V.P. test, an orange color, which may be intensified by  $\alpha$ -naphthol, develops upon addition of potassium hydroxide to positive cultures.

A number of solid media have been devised for isolation and recognition of enteric bacteria, two of which are commonly used for isolation of coliform organisms. On **Endo's agar** (nutrient agar containing lactose and an indicator, basic fuchsin decolorized with sodium sulfite) colonies of *Bact. coli* are typically deep red in color with a metallic sheen and reddening of the surrounding medium. The red color results from the reaction of acetaldehyde (a product of lactose fermentation) with the sodium sulfite of the indicator. On **eosin-methylene blue agar** (E.M.B. agar: peptone, lactose agar containing yellow eosin and methylene blue dyes) colonies of coliform bacteria are also dark in color with a metallic sheen. In this instance the staining of the colonies is attributed to acid formation, which permits adsorption of the dye. On these media colonies of *Bact. aerogenes* are colored, although less intensely than those of coliform bacteria.

**Types of *Bacterium Coli*.** The observation of differences in fermentation reactions of strains of *Bact. coli* has led to recognition of several types or varieties. Approximately one-half of cultures, named *Bact. coli communis*, do not ferment sucrose and are separated from the sucrose-fermenting strains, known as *B. coli communior*. These varieties are in other respects typical lactose-fermenting *Bact. coli*. The ability to ferment lactose is also subject to variation. In a study of this property of the colon bacilli has contributed greatly to knowledge of bacterial variation. Some strains (anaerogenic type) ferment lactose with production of acid but not gas. *Bacterium coli mutabile* is interesting in that young cultures fail to ferment lactose and produce pale colonies on different media, whereas after some days papillae appear on these atypical colonies, organisms from which show typical reactions on Endo's and eosin-methylene-blue agar and are found to ferment lactose. The parent bacteria during growth and cell division thus produce offspring different from the parent in the ability to ferment lactose.

**Pathogenicity.** Within the intestinal tract *Bact. coli* is a harmless member of the intestinal flora. Some strains have, however, been incriminated in many diarrheal diseases. Infections elsewhere in the body with *Bact. coli* may be severe and highly fatal. *Bacterium coli* is a frequent cause of infections of the urin-



such as cystitis and pyelitis, and is a less frequent cause of puerperal fever, enteritis and wound infections. Meningitis and septicemia are unusual, but the disease may be particularly serious in infants.

**chemotherapy.** *Bacterium coli* is sensitive to the sulfonamide drugs, particularly sulfathiazole and sulfadiazine, and to streptomycin, both of which are effective against infections with this organism. Aureomycin and chloromycetin may also be of value.

**Bacterium Aerogenes.** *Bacterium aerogenes* is widely distributed in nature, in plants, grains and in the intestinal contents of man and animals. The medical and sanitary importance of *Bact. aerogenes* stems from its close resemblance to *B. coli* and its wide distribution. It may at times be present in water without causing fecal pollution, a fact which may necessitate differentiation of these organisms. Typical *Bact. aerogenes* is generally not pathogenic, although a related *Bact. cloacae* has been associated with respiratory infections known as legionnaires fever and grain fever.

*Bacterium aerogenes* is similar morphologically and culturally to *Bact. coli*. The important differences between these organisms are included in Table 12. Growth is better at temperatures below 30° C, indole and hydrogen sulfide are not produced, the methyl red test is negative, the Voges-Proskauer reaction is positive and growth occurs in citrate medium. Many carbohydrates are fermented. *Bacterium cloacae* is a related lactose-fermenting encapsulated bacterium, which is distinguished from the *coli-aerogenes* group in the liquefaction of gelatin. Colonies are gray-white or pigmented yellow.

**The Paracolon Bacteria and Colon-Aerogenes Intermediates.** A great number of cultures of lactose-fermenting gram-negative bacteria are not typical *B. coli* or *Bact. aerogenes*. One such group, the members of which ferment lactose readily, is known as **colon-aerogenes intermediates**. On the basis of indole, methyl red, Voges-Proskauer and citrate tests (IMViC tests) almost all possible combinations of physiological characteristics from typical *Bact. coli* and typical *Bact. aerogenes* have been recognized.

The paracolon bacteria represent another heterogeneous and atypical group of lactose-fermenting organisms. The paracolon bacteria ferment lactose irregularly and slowly after cultivation for some days. Some fail to produce gas in glucose and a few strains produce only acid in other sugars. From the table it is evident that such cultures may be confused with the *Salmonella* and dysentery types of bacteria. A number of groups of paracolon bacteria are recognized on the basis of IMViC reactions and motility.

Atypical coliform organisms, both intermediate and paracolon types, are widely spread in nature, in water, soil, milk and the feces of man and animals. They have been associated with outbreaks of gastro-enteritis, largely on circumstantial evidence. However, occasional laboratory infections have occurred which leave little doubt of the pathogenicity of some strains.

**Immunology.** Immunological studies of the *coli-aerogenes* group have not been of value in their identification. On the basis of agglutination tests the

group is composed of a variety of immunological types which do not correspond to physiological groups. Furthermore, coliform bacteria are related immunologically to Friedländer's bacillus, the dysentery group, and they possess antigens common with a number of bacteria of the *Salmonella* group.

### THE FRIEDLÄNDER GROUP

There is a group of heavily encapsulated bacteria closely related to the *aerogenes* group, the best known representative of which is the bacterium isolated by Friedländer (1883) from pneumonia. This organism (Friedländer's bacillus, *Klebsiella pneumoniae*, *Bacterium pneumoniae*, *Bacillus pneumoniae*) is known as the causative agent of pneumonia and other infections of the air

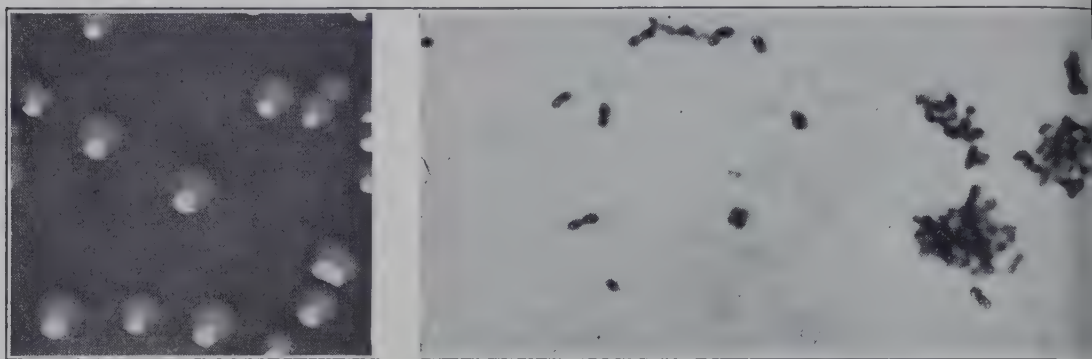


Fig. 160. (Left) Colonies on blood agar and (right) photomicrograph (magnification approximately  $\times 1,400$ ) of *Klebsiella pneumoniae* (Friedländer's bacillus). Note mucoid appearance of the colonies.

sages, although it and related forms may infect other regions of the body. Members of the group are heavily encapsulated and produce large mucoid colonies. Related or identical bacteria include the ozena bacillus, the bacillus of rhinoscleroma, both pathogens of the nasal passages, and *Bacillus mucosapapulatus*.

**Morphology.** Friedländer's bacillus is a nonmotile, gram-negative bacterium; its elongated coccobacillary form, the arrangement of the cells in pairs and the presence of a capsule cause it closely to resemble the pneumococcus. In cultures, longer bacteria are commonly seen. The bacteria are readily stained by ordinary dyes.

Surface colonies of encapsulated strains are large (several millimeters in diameter in 24 hours), sticky, glistening and mucoid, whereas those of nonencapsulated cultures resemble those of *Bact. coli*. There is no hemolysis of blood.

Variation from the encapsulated mucoid type of organism to the nonencapsulated forms is analogous to the S→R variation, and occurs readily in culture on artificial media. Variants tend to be unstable. Cultivation on media containing blood, serum or glucose favors persistence of the mucoid characteristics.

**Metabolism.** Members of the group are aerobic, facultatively anaerobic and



best at 37° C. The organisms are readily killed by heat and disinfectants and are somewhat resistant to drying.

Typical biochemical reactions of the group have been included in Table 12. A number of strains fail to ferment lactose and sucrose or do so only after some time. Other reactions, such as production of indole, coagulation of milk and the indole and Voges-Proskauer reactions, are likewise not constant. The biochemical reactions do not provide an adequate basis for classification.

The Friedländer bacteria grow well in many laboratory media and will develop on synthetic media containing ammonium sulfates, phosphates and a fermentable carbohydrate such as glucose, without addition of vitamins. Capsule formation is induced by carbohydrate media, which soon become sticky and viscous due to the accumulation of capsular material.

**Immunology.** Like the pneumococci, members of the Friedländer's group may be divided into types by means of specific capsular antigens and possess a common cell antigen within the cells. The capsular antigens are polysaccharides, similar to those of the pneumococcus capsules. By means of agglutination and precipitation reactions similar to those with pneumococci, types A, B and C and a relatively small type X of encapsulated Friedländer's bacilli are commonly recognized. Type B is related immunologically to Type II pneumococcus. Immune serum against a specific organism will protect against the experimental infection with the same type of

organism. The *ozena bacillus* appears to be unrelated to the Friedländer types, as the rhinoscleroma organism reacts with Friedländer antiserum.

**Pathogenicity.** The Friedländer group is best known as the cause of respiratory infections. Pneumonia due to Friedländer bacillus is particularly severe and involves the upper respiratory tract and the bronchi tend to be chronic. In addition, either identical or related encapsulated organisms (often named *B. capsulatus*) are sometimes cultivated from infections of the urinary tract and form abscesses. Rarely, members of the Friedländer group are isolated from the spinal fluid in meningitis. Natural infections, particularly pneumonias, occur in lower animals, and a high fatality results from experimental infections. Type A is most frequently isolated from human infections.

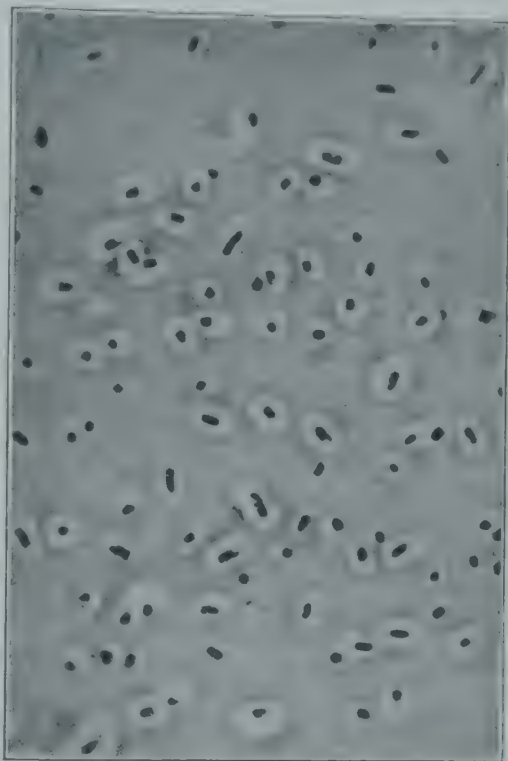


Fig. 161. Photomicrograph of smear of a member of the Friedländer group of bacteria stained to show the large capsules surrounding the bacterial cells. (Magnification approximately  $\times 1,400$ .) (Kral.)

**Chemotherapy.** Friedländer organisms are relatively insusceptible to sulfonamide drugs and are highly resistant to penicillin. They are, however, naturally susceptible to streptomycin, aureomycin and chloromycetin.

### THE SALMONELLA GROUP

The Salmonellae are a group of enteric bacteria parasitic and pathogenic to man and animals. The most important human pathogen of the group is *Salmonella typhosa* (*Bacterium typhosum*, *Eberthella typhi*), described in 1880 by Eberth and isolated in 1884 by Gaffky from typhoid fever. In 1886 Salmon and Smith isolated *S. cholerae suis* from animals and in 1888 Gaertner demonstrated the presence of *S. enteritidis* in gastro-enteritis. In addition to typhoid fever, *Salmonella* species have been associated with human paratyphoid fever and gastro-enteritis and with similar infections in animals. None of the salmonellae are saprophytic.

Members of the group are motile or nonmotile, gram-negative rods which ferment dextrose but not lactose. With few exceptions gelatin is not liquefied and indole is not produced. The Voges-Proskauer test is negative.

**Morphology.** The salmonellae are indistinguishable morphologically from other members of the enteric group. They do not have capsules and, with the exception of *S. pullorum* and *S. gallinarum*, all members of the group are motile by means of peritrichous flagella.

The colonies on ordinary media are gray-white in color, 1 to 2 mm. in diameter after 24 hours' incubation and are smooth and entire or have slightly indented margins. The colonies are colorless on Endo's and eosin-methylene blue agars.

Smooth to rough variation occurs in the *Salmonella* group, and in addition there is a variation in antigenic structure known as phase variation, which is related to colonial form, virulence, etc. (see below).

**Physiology.** Members of the group develop readily on ordinary media with or without either the presence or absence of oxygen. The optimum temperature for growth is 37° C. These bacteria are not unusually resistant to heat or chemical disinfectants and are destroyed by pasteurization and usual methods for sterilization. They may, however, survive for considerable periods of time in water and in the laboratory on artificial media. Growth requirements are not exacting; development having been attained in synthetic media without the addition of vitamins.

Biochemical reactions of differential value are indicated in the accompanying tables. In addition, maltose, mannitol, dulcitol, sorbitol and dextrin are usually attacked. It is important to note that *S. typhosa* differs from most species in its failure to produce gas in carbohydrate-containing media. Although biochemical reactions have value in characterization of the group as a whole, immunologic tests (agglutination) are the basis of identification of species.

**Differential Media.** The isolation and differentiation of *Salmonella* cultures from the coliform group of bacteria is of practical importance in recognition



tion and the carrier state. On Endo's and eosin-methylene-blue agars these organisms produce colorless colonies. They may, however, be present in insufficient numbers to be separated from the coliform bacteria. Media inhibitory to coliform group are, therefore, often utilized for isolation of colonies. **Desoxycholate-citrate agar** contains sodium desoxycholate, sodium citrate, lactose, and phenol red indicator in an infusion medium base. Coliform organisms are considerably inhibited and produce red colonies, whereas *Salmonella* and dysenteriae organisms produce colorless colonies. **Salmonella-Shigella (S-S) agar** is a commonly used medium containing bile salts, sodium citrate, lactose, brilliant green and phenol red. Colonies of lactose-fermenting bacteria are small and have a red color. Wilson and Blair bismuth sulfite agar is of particular value in isolating *S. typhosa*, colonies of which are typically black. Preliminary enrichment in a liquid medium, such as selenite-F, which is inhibitory to coliform organisms, is sometimes employed prior to plating for the isolation of small numbers of enteric pathogens. Following isolation, cultures are identified by biochemical and immunological tests.

**Antigenic Structure.** The antigenic structure of the bacterial cell provides the basis for classification of *Salmonella*. These bacteria contain a number of different antigens, some of which, the **somatic or O antigens**, are associated with the bacterial cell substance, and others, the **flagellar or H antigens**, are present in the flagella. These antigens have different properties, so that they can be separated from each other; and the antigens of different bacteria may be compared by the agglutination technique.

The terminology of the O and H antigens is derived from the German descriptions of the colony form of motile and nonmotile *Proteus* cultures. Thus, the **H antigens** (from Ger., *Hauch*) are present in the flagella of motile cultures, and tend to form a veil or film about colonies on a solid medium; and the **O antigens** (Ger., *ohne Hauch*) are present in cells of both motile and nonmotile cultures, although the latter do not produce spreading growth.

The somatic or O antigens are by agreement named by Roman numerals. The antigenic structure of a cell may then be shown by a series of numerals. *S. paratyphi A* contains O antigens I, II, XII (Table 13). The O antigens are resistant to boiling, alcohol and acid and may be prepared from motile bacteria by heating the cells suspended in alcohol. They are denatured by formalin. O agglutination is characteristically finely granular. The H antigens are designated by the small letters of the alphabet and by Arabic numerals. The H antigens are destroyed by heating, alcohol and treatment with acid, but are resistant to formalin. H agglutination produces large flaky clumps of the bacterial cells.

The use of both lower case letters of the alphabet and Arabic numerals to designate H antigens is arbitrary but results from a type of variation known as **phase variation**. Phase variation refers to the possession of different flagellar antigens by cells of the same culture. The letters of the alphabet indicate specific flagellar antigens of Phase I, the numerals nonspecific antigens of Phase II. Many

TABLE 13. DIFFERENTIAL REACTIONS OF REPRESENTATIVE SALMONELLA SPECIES \*

IMMUNO-LOGIC GROUP	SPECIES	ANTIGENIC FORMULA			BIOCHEMICAL REACTIONS							
		O ANTIGENS (SOMATIC)	H ANTIGENS (FLAGELLAR)		GAS FROM CARBO-HYDRATES	DEX-TROSE	XYLOSE	ARAB-INOSE	TREHA-LOSE	INOS-ITOL	D-TAR-TRATE	PRO-DUCE H <sub>2</sub> S
			SPECIFIC PHASE I	NON-SPECIFIC PHASE II								
A	<i>S. paratyphi A</i>	I, II, XII	a		+	—	+	+	+	—	—	±
B	<i>S. paratyphi B</i>	(I) IV, (V) XII	b	I, 2	+	±	+	+	+	±	±	+
	( <i>S. schottmüllerii</i> )	(I) IV, (V) XII	i	I, 2, 3	+	+	+	+	+	±	±	+
	<i>S. typhi-murium</i>	IV, XII	f, g	e, n, x	+	+	+	+	+	—	—	+
	<i>S. abortus-equi</i>	(I) IV, XII			+	+	+	+	+	+	+	+
C-1	<i>S. derby</i>	(I) IV, XII			+	+	+	+	+	+	+	+
	<i>S. paratyphi C</i>	VI, VII	c	I, 5	+	+	+	+	+	—	—	+
	( <i>S. hirschfeldii</i> )	VI, VII	c	I, 5	+	+	+	+	+	—	—	—
	<i>S. cholerae-suis</i>	VI, VII	m, t		+	+	+	+	+	—	—	—
C-2	<i>S. Oranienburg</i>	VI, VII	e, h	I, 2, 3	+	+	+	+	+	—	—	—
	<i>S. Newport</i>	VI, VIII			+	+	+	+	+	—	—	—
D	<i>Salmonella typhosa</i>	IX, XII	d		—	±	±	+	+	—	—	+
	( <i>Bact. typhosum</i> )	(I) IX, XII	g, m		+	+	+	+	+	—	—	+
	<i>S. enteritidis</i>	I, IX, XII	I, v	I, 5	+	+	+	+	+	—	—	+
	<i>S. Panama</i>	IX, XII			+	+	+	+	+	—	—	+
E	<i>S. gallinarum</i> †				+	+	+	+	+	—	—	+
	<i>S. London</i>	III, X, XXVI	I, v	I, 6	++	+	+	+	+	+	+	+
	<i>S. anatum</i>	III, X, XXVI	e, h	I, 6	++	+	+	+	+	+	+	+
	<i>S. Newington</i>	III, XV	e, h	I, 6	++	+	+	+	+	+	+	+
F	<i>S. Senftenberg</i>	I, III, XIX	g, s, t		++	+	+	+	+	+	+	+
	<i>S. Worthington</i>	I, XIII, XXIII	I, w	z	++	+	+	+	+	+	+	+
	<i>S. Onderstepoort</i>	(I), VI, XIV, XXV	e, h	I, 5		++	++	++	++	+	+	+



ies possess antigens of both phases, whereas others have but a single phase. This variation is an inherited characteristic of the bacteria. Although cells in Phase I and Phase II may be separated, it is normal for some cultures (diphasic) to be comprised of both types of cells at the same time.

The *Salmonella* group is divided into a number of immunological groups comprised of species which have O antigens in common. Thus *S. paratyphi A* possess O antigens I, II and XII, and the members of group B possess O antigens VI, VII and VIII are characteristic of group C, etc. The combinations of the O and H antigens characterize the bacterial species. For example, the antigenic formula of *S. typhosa* is IX, XII; d; —, and *S. paratyphi A* (I) IV, (V); XII b; 1,2.

**Pathogenicity.** The salmonellae are widely distributed parasites and pathogens of man and the higher animals. Indeed, ingestion of meat from infected animals or other contaminated food is a frequent source of human infection with many *Salmonella* species. *Salmonella enteritidis* and *S. typhi-murium* are common in wild rats and mice, *S. cholerae-suis* is found in swine and *S. gallinarum* is a parasite of domestic fowl. There is, however, little specificity among the species of animals which may be infected with many Salmonellae, the organisms often being found naturally in man and domestic animals. On the other hand, *S. typhosa*, *S. paratyphi A* and *S. paratyphi B* are almost exclusively human parasites and pathogens.

Salmonellae do not produce a true exotoxin, but the cells are highly toxic following injection of experimental animals. This endotoxic substance appears to be a polysaccharide-lipoid complex.

**Disease in Animals.** Rats and mice, horses, swine, domestic fowl and, to a small extent, cattle are naturally infected with salmonella. Among rodents, typhoid disease with diarrhea, septicemia and abscesses or necrosis of the liver and spleen is produced by *S. enteritidis* and *S. typhi-murium*. This disease is at times highly fatal and epidemic, but infection is also chronic or without symptoms (*i.e.*, the carrier state). In swine *S. cholerae-suis* is the cause of an enteric infection. Salmonella infection (*S. abortus-equi* and *S. abortus-ovis*) is one cause of abortion in horses and sheep. Infections with *S. pullorum* and *S. gallinarum* are important causes of a diarrheal disease in fowl which may reach epidemic proportions. Of particular importance is the presence of salmonella in eggs from naturally infected hens, and in recent years a wide variety of salmonellae has been isolated from eggs and fowl, occasionally in association with outbreaks of avian gastro-enteritis.

Animal diseases may be experimentally reproduced by inoculation of pure cultures of the animal pathogens. It is of importance that typhoid fever has not been reproduced in animals, inoculation of *S. typhosa* being followed by the development of a nonspecific febrile disease which does not resemble the human infection.

**Pathogenicity for Man.** In man Salmonellae produce two principal types of disease: acute gastro-intestinal infection or *Salmonella* food poisoning and

the enteric fevers, typhoid fever and paratyphoid fever. These two diseases differ in their clinical, epidemiological and bacteriological characteristics. A small percentage of meningitis, liver and bone abscesses, septicemias and other infections are caused by salmonellae.

**Salmonella Food Poisoning.** *Salmonella enteritis* is an acute, febrile diarrheal infection caused by various members of the *Salmonella* group. The infection is generally mild, although it may be severe, and is acquired by ingestion of food containing the organisms. The onset of symptoms usually begins in 6 to 24 hours following ingestion of the contaminated food. The diagnosis of *Salmonella* food poisoning is generally made upon isolation of salmonellae from the stools of patients having the typical disease. Other forms of food poisoning, particularly staphylococcal food poisoning, should be excluded by appropriate diagnostic methods. Isolation of organisms from suspected food provides additional evidence of the causative role of the bacteria and should be carried out whenever possible. Careful clinical and bacteriological evidence should be obtained, since salmonellae are not infrequently found in the stools of healthy individuals, particularly in institutions where salmonella harborage presents many of the same problems as institutional dysentery. Furthermore, the pathogenicity for many of the *Salmonellae* is poorly established.

The types of *Salmonella* most frequently identified from outbreaks of acute enteritis in the United States between 1934 and 1941 are *S. typhi-murium* (*aertrycke*), *S. Newport*, *S. Panama*, *S. Oranienburg*, *S. enteritidis*, *S. anatum* and *S. Sandiego*. *Salmonella pullorum* has recently been isolated from man, although its pathogenicity is not clearly established. The epidemiological aspects of *Salmonella* food poisoning are discussed in Chapter 43.

### TYPHOID AND PARATYPHOID FEVERS

Human infection with *S. typhosa*, *S. paratyphi A*, *S. paratyphi B* and *S. paratyphi C* usually results in a prolonged enteric fever. These diseases, known as typhoid fever and paratyphoid fever, differ in their specific causative agents, but are quite similar in epidemiology and clinical aspects. In general, typhoid fever is the more severe and more frequent disease. The infections are for practical purposes limited to man, although the paratyphoid organisms are occasionally isolated from animals, in which case human infection may be acquired by association with infected animals.

Typhoid fever usually begins suddenly approximately ten days to two weeks after infection and is characterized by a continuous fever, intestinal symptoms, general weakness and malaise and a rose-colored skin eruption. During the acute illness, the intestinal wall is commonly ulcerated and the lymph follicles and nodes of the intestine and abdominal cavity are infected and acutely inflamed. The spleen is enlarged.

The bacteriology of typhoid fever has been subject to extensive investigation. Following infection the organisms are thought to invade the abundant lymphoid



of the intestinal wall (Peyer's patches) and to multiply in these and in lymph nodes. Typhoid bacilli are widely distributed throughout the body tissues and in the blood during the first 7 to 10 days of the disease. Invasion of the intestinal wall is commonly followed by ulceration of the mucous membrane and discharge of large numbers of bacteria in the feces. With the development of immunity, localization and destruction of the bacteria occur, with sterilization of the blood and healing of the intestinal lesions. The bacteria may remain present during and after convalescence in many areas of the body, most important of which are the spleen, the gallbladder and the urinary tract. In the latter instances discharge of bacteria into the feces and urine contributes to the transmission of the bacteria. Complications, such as meningitis



Fig. 162. (Left) Colonies on blood agar and (right) photomicrograph (magnification approximately  $\times 1,400$ ) of *Salmonella typhosa*.

abscesses, sometimes result from persistence of infection in the central nervous system, the bones and other tissues, and hemorrhages and perforation of the intestinal tract with peritonitis may develop during the acute stages. The period of disability from typhoid fever is commonly several weeks.

**Salmonella Typhosa** (*Bacterium typhosum*, *Eberthella typhi*, the typhoid bacillus). Characteristics of *S. typhosa* have already been described. The antigenic formula of O and H antigens is IX, XII; d; —. In addition, this bacterium possesses another cellular antigen which is found particularly in freshly isolated cultures. This antigen, known as **Vi antigen** (virulence) is heat-labile and stimulates production of specific antibody. Vi antibody appears to be particularly important in protection against infection with *S. typhosa*. Vi antigen is present in a high proportion of strains of *S. typhosa*, and the same antigen is found in few other *Salmonellae*.

The bacteriological diagnosis of typhoid fever is made by isolation of *S. typhosa* from the blood, urine and feces of patients. Blood cultures are positive during the first week or ten days, whereas stool and urine cultures are generally positive after the first week. Stool cultures should be examined by identification on differential media (desoxycholate-citrate, S-S, Wilson and Endo's or EMB agars). Typhoid carriers are also recognized by isolation of *S. typhosa* from specimens of urine or feces. Bacteriophage typing of

strains of *S. typhosa* has been used in epidemiological studies. By this method it is possible to recognize a number of otherwise indistinguishable types of *S. typhosa* and to trace the spread of infection with one type.

**Immunity.** Convalescence from typhoid fever is associated with increased but incomplete immunity to a second attack of the disease which is related to the presence of specific antibodies in the blood. Although bacteriolysins and opsonins may be present, the agglutination or **Widal test** is most commonly used to demonstrate antibodies.

The increase in agglutinin titer of the blood observed during the course of typhoid fever has diagnostic importance. The agglutinin titer (Widal reaction) is commonly increased in the second and usually by the fourth week of the disease. Both H and O agglutinins are demonstrable by tube or slide agglutination methods. Usually an H agglutinin titer of a 1:100 or 1:200 dilution of serum and an O agglutinin titer of 1:100 are significant. In vaccinated persons, however, the presence of a positive Widal reaction must be interpreted cautiously, a persistent rise in titer being of greatest significance. Agglutinins continue to be present in the blood for many months or years following the disease.

**Vaccination against Typhoid Fever.** Vaccination against typhoid fever has proved highly successful in the prevention of the disease and is a valuable method of control (see below). Ordinarily, heat-killed suspensions of *S. typhosa*, often combined with *S. paratyphi A* and *S. paratyphi B*, are used. The vaccine is standardized to contain 1,000 million cells of *S. typhosa* and 750 million cells of each of *S. paratyphi A* and *S. paratyphi B*, is administered in three weekly intramuscular injections of 0.5, 1.0 and 1.0 ml. Following immunization antibodies against the bacteria appear in the blood, and there is increased resistance to infection. Although it provides protection against ordinary exposure, the resistance is not complete, and following massive exposure immunized persons sometimes develop mild typhoid fever. Both O and H antibodies may be demonstrated following vaccination. It appears to be important that fully virulent *S. typhosa* be used in the vaccines, a matter which has received considerable attention, as has the entire subject of vaccination and prevention of typhoid fever, by the U. S. Army Medical School. A satisfactory immune serum has not been developed.

**Epidemiology of Typhoid and Paratyphoid Fevers.** Typhoid and paratyphoid fevers are among the most important communicable diseases of man. In times past they have occurred in extensive epidemics in cities, among armies in hospitals, asylums and in families in all the major continents of the world. Although at the present time large epidemics are infrequent in temperate climates, the disease remains endemic and may become epidemic. Typhoid and paratyphoid fevers continue to be important causes of illness in areas where sanitation is poor. In the United States the median number of cases reported annually between 1936 and 1940 was 14,903 and in 1943 over 5,000 cases were reported. At the present time the death rate is ordinarily less than 1.0 per 100,000 population, a figure which represents an improvement of over 95 per cent since 1900. Indeed, the number of both cases and deaths has been remarkably reduced.



ing recent years, largely as a result of improved sanitation of water, food and . The diseases occur during all months of the year but are particularly frequent during the late summer and early fall.

**Sources of Infection.** As has been indicated, these bacteria are almost exclusively parasites of man and may leave the body in the feces or in the urine. In patients with the active disease and healthy carriers are sources of infection for other members of the population. During the acute infection patients commonly discharge bacilli for several weeks, and a small proportion (convalescent carriers) continue to have positive cultures for several months. The chronic carrier state, on the other hand, represents a prolonged infection, with discharge of bacteria. Fecal carriers, in whom infection is commonly localized in the gall bladder, are much more frequent than urinary carriers with localization in the urinary bladder. The typhoid carrier is usually of the intermittent type, that is, cultures of the feces or urine may be positive at one time and negative at others. The number of carriers of typhoid bacteria within the general population is not known. However, careful investigation of epidemics frequently reveals a carrier to be the source of infection.

**Transmission of Infection.** Infection with the enteric fevers is acquired by ingestion of the bacteria, usually in water or food contaminated with feces or urine of infected patients or carriers. The most important methods of transmission are, therefore, contaminated water, food and milk. These are responsible for the epidemics of the disease. In addition, organisms may be spread by domestic contacts, contaminated hands and personal contact. The spread of typhoid fever indicates inadequate sanitation, either community or personal.

**Control.** Control of the enteric fevers and diarrheal diseases by community sanitation of water, food and milk (see Chapter 43) has been followed by spectacular reduction in the amount of disease, particularly in urban areas. Indeed, sanitation is the primary method of control in civilian populations, large-scale vaccination having proved unnecessary in most instances. However, vaccination has great importance in the prevention of disease among armies and during catastrophes when there is interruption of sanitary facilities. Preventive immunization is also recommended for travelers in rural areas and in countries where sanitation is relatively primitive. In order to reduce the possibilities of transmission, patients with the disease are required to be isolated, and known carriers are subject to supervision by the health department. It is usually unnecessary to isolate carriers of infection, but they should not be employed in the food industry.

The application of available methods of sanitation and prevention has resulted in remarkable reduction, but not in the elimination, of typhoid fever. A small number of cases remaining in the presence of adequate community sanitation is referred to as **residual typhoid fever**, and is usually encountered in the form of small local and sporadic epidemics often traceable to a previously recognized carrier of the infection. The eradication of residual typhoid fever

is a particularly difficult epidemiological problem which demands continuous effort by the health department.

**Chemotherapy.** Penicillin is ineffective in typhoid fever and results with streptomycin have been disappointing and inconclusive, although *S. typhosa* is moderately susceptible to streptomycin *in vitro*. Drugs of the sulfonamide series are relatively ineffective. Aureomycin and chloromycetin are effective.

### THE DYSENTERY GROUP

Dysentery has been one of the most important communicable diseases. Although although notably less frequent at the present time, it continues to be prevalent in tropical countries and wherever poor hygienic conditions exist. Dysentery is an infection of the intestinal tract characterized by diarrhea with blood in the stool, abdominal pain and fever. Dysentery may result from infection with

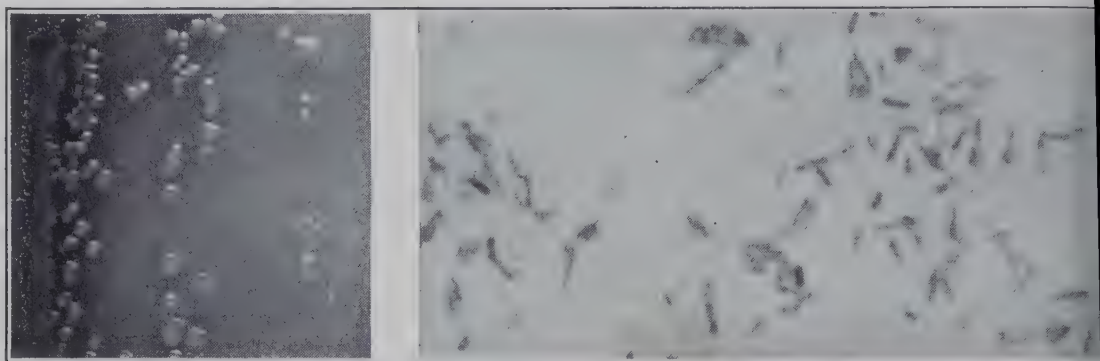


Fig. 163. (Left) Colonies and (right) photomicrograph (magnification approximately  $\times 1,400$ ) of *Shigella paradysenteriae*, Flexner type of the dysentery bacillus.

amebae (amebic dysentery), but more commonly it is caused by members of the dysentery group of bacteria (bacillary dysentery). Shiga first demonstrated the role of bacteria in dysentery when he isolated the organism known as the Shiga bacillus during an epidemic in Japan in 1898. This bacillus was specifically agglutinated by the serum of patients having the disease. In 1900 Flexner and Strong both described related bacilli from similar epidemics in the Philippine Islands, and Kruse in Germany identified Shiga's bacillus from dysentery. Since these early reports a number of different, but related, bacteria have been clearly associated with epidemic and sporadic or endemic dysentery in many parts of the world.

The dysentery bacilli are classified together as a subgroup of the enteric bacteria, commonly in the genus *Shigella*. Although they possess many distinct properties, the dysentery bacteria are related to the *Salmonella*, including *Salmonella typhosa*, and to the *coli-aerogenes* groups of bacteria.

**Morphology.** The dysentery bacteria cannot be distinguished morphologically from the other enteric bacteria. All members of the group are nonmotile, gram-negative rods. The colonies on ordinary nutrient media are smooth, gray



somewhat granular and become approximately 2 mm. in diameter in 24 hours. Variation of the S  $\rightarrow$  R type is frequently observed, and other variants as mucoid cultures are sometimes encountered.

**Physiology.** Members of the group develop either aerobically or anaerobically at 37° C. They are not unusually resistant to heat or disinfectants and are destroyed by pasteurization and the ordinary methods of heat sterilization and disinfection. The organisms grow well on ordinary bacteriological media and may be cultivated in synthetic media. The majority of strains requires amino acids and nicotinic acid for growth.

The biochemical reactions, particularly the fermentation reactions, are of great value in classification of the group. All members of the group ferment dextrose with acid production, only the Newcastle bacillus producing gas; on the basis of fermentation of mannitol the Shiga bacillus and the Schmitz bacillus may be distinguished from *Shigella paradysenteriae* and related forms. The important differential characteristics are indicated in Table 14.

**Differential Media.** Because of their pathogenicity, the isolation and recognition of the dysentery group is of great practical importance, and several differential media have been devised for the separation of this as well as the *Shigella* group from the *coli-aerogenes* group. On Endo's and eosin-methylene blue agars, the colonies are colorless. However, on these media the abundance of the *coli-aerogenes* group often creates great difficulty in isolation of small numbers of pathogens from stool cultures. As in the case of the *Salmonella*, the media of greatest value are desoxycholate-citrate and *Salmonella-Shigella* media. These media should be heavily inoculated with specimens being examined for dysentery bacilli and typical colonies isolated for further study. Identification is accomplished by cultural studies and agglutination reactions.

**Classification.** Classification of the dysentery group presents some difficulty. However, the following groups or species are commonly recognized:

*Shigella dysenteriae* (*Bacterium shigae*, the Kruse-Shiga bacillus): The first member of the group to be described, the Shiga bacillus is distinctive in its failure to ferment mannitol and produce indole. It is antigenically homogeneous, one typical type being recognized. The Shiga bacillus is the only member of the group which produces a typical exotoxin.

*Shigella ambigua* (*Bacterium ambiguum*, the Schmitz bacillus): *Sh. ambigua* differs from *Sh. dysenteriae* antigenically and in the fermentation of rhamnose and production of indole. Exotoxin is not formed.

*Shigella paradysenteriae* (*Bacterium flexneri*, the Flexner bacillus): The latter group is characterized by fermentation of mannitol and failure to ferment sucrose, xylose, dulcitol and rhamnose. Indole is produced. A number of antigenically different types of *Sh. paradysenteriae* are recognized; these are differentiated by means of the agglutination and agglutinin-absorption reactions. The best known system of typing is that of Andrews and Inman, which recognizes five types (V, W, X, Y and Z) on the basis of four antigens. V, W, X and Y. More recently the Boyd classification has become widely used. According

TABLE 14. BACTERIA OF THE DYSENTERY GROUP

SPECIES	FERMENTATION								INDOLE	TOXIN PRODUCTION	IMMUNOLOGICAL TYPES
	DEXTROSE	LACTOSE	SUCROSE	MANNITOL	DULCITOL	SORBITOL	RHAMNOSE	XYLULOSE			
<i>Bact. shigae</i>	+	—	—	—	—	—	—	—	—	+	One principal type; cross-reactions with <i>B. ambigua</i>
<i>Bact. ambigua</i> Schmitz bacillus	+	—	—	—	—	—	+	—	+	—	One type; cross-reactions with <i>B. shigae</i>
<i>Bact. flexneri</i>	+	—	—	+	—	—	+	—	+	—	Types V, W, X, Y and Z (Andrewes and Inman); Types I, II, III, IV, V, VI, etc. (Boyd)
<i>Bact. alkalescens</i>	+	—	+	+	+	+	+	+	+	—	Types I (original), II and III
<i>Bact. sonnei</i>	+	+	+	+	—	—	+	—	—	—	Types I and II
<i>Bact. dispar</i>	+	+	+	+	—	+	+	+	+	—	Several types; cross-reactions with <i>B. flexneri</i>
Newcastle bacillus	+	—	—	+	+	—	—	+	—	—	One type; cross-reactions with <i>B. flexneri</i> , Type VI



is schema, six major types (I to VI), which include the Andrewes and n types as well as newly recognized varieties, are separated by means of specific agglutination tests. The members of the Flexner group have thus found to contain type specific antigens. They contain a group specific en as well.

*Shigella alkalescens* (*Bact. alkalescens*): *Shigella alkalescens* is an uncommon of dysentery. It may be distinguished from other members of the group biochemical reactions and by agglutination tests with specific antiserum.

*Shigella sonnei* (*Bact. sonnei*): *Shigella sonnei* is a lactose-fermenting ism, although the fermentation occurs only after some days' incubation. bacterium may be recognized by the fermentation of lactose and mannitol and agglutination tests. Two types (Types I and II) of *Sh. sonnei* are recognized immunological methods: they possess the same antigens, but in different ortions.

*Shigella dispar* (*Sh. madampensis* and *Sh. ceylonensis*): *Shigella madampensis* *Sh. ceylonensis* are lactose-fermenting dysentery bacilli which differ in the entation of dulcitol and which together are considered here as *Sh. dispar*. bacteria are antigenically heterogeneous.

*The Newcastle bacillus*: The Newcastle bacillus differs from other members e group in that it is feebly motile and produces a small amount of gas from ohydrates. By agglutination tests the organisms are of one type, but they closely related to *Sh. paradysenteriae* Type VI.

**Pathogenicity.** The dysentery bacilli are natural parasites and pathogens an but, with the exception of monkeys in captivity, natural infections are known in animals. Indeed, laboratory studies have shown that bacillary entery may be reproduced only in the monkey and that this animal is naty highly resistant to experimental infection. It is interesting that monkeys a diet deficient in Vitamin M (folic acid) are more susceptible to bacillary entery than are normal monkeys. Certain members of the group, particularly *S. shigae*, are highly toxic and virulent when injected into rabbits and mice. role of the dysentery group of bacteria in human dysentery is, however, ly shown by the association of the bacteria with epidemics of the disease, erimental and accidental human infections and development of specific utinins by persons suffering from infection. The bacteria are present in large bers in the intestinal contents during the acute disease and may be the pre- inant organism in stool cultures. Invasion is limited to the intestinal tract, hich intense inflammation with ulceration and bleeding is commonly pro- ed. Cultures of the blood are negative for dysentery bacilli.

The diagnosis of bacillary dysentery is established by isolation and identifica- of the specific causative organism. A rise in titer of specific agglutinins in blood provides confirmatory evidence. For isolation of bacteria, fecal speci- s or rectal swabs are cultured on selective media, a procedure which permits gnition of carriers as well as of acute infections.

**Toxin Production.** *Shigella dysenteriae* is the only member of the genus which produces a true exotoxin (neurotoxin). This toxic substance is present in filtrates of cultures incubated for several weeks, is heat-labile and may be detoxified with formalin. In experimental animals the toxin affects the central nervous system, producing paralysis and death. In addition to toxicity of exotoxin, an endotoxin is present in the Shiga bacillus. This material, which chemically is a polysaccharide-lipid-polypeptide complex, analogous to that isolated from certain other enteric bacteria, appears to affect chiefly the gastrointestinal tract. A similar endotoxin appears to be present in *Bact. flexneri* which is also highly toxic to laboratory animals.

**Epidemiology.** The diarrheal diseases are frequent causes of disability in temperate and particularly in tropical countries. The mortality rate is relatively low, but diarrhea is an important cause of death among infants. Reported cases represent only a fraction of the number of existent infections, which is estimated to be many hundreds of thousands of cases per year. As indicated in the introduction, diarrhea may result from many causes, of which specific infections such as amebic dysentery, paratyphoid infections and bacillary dysentery are the most important. With better methods of isolation and identification of the bacteria, bacillary dysentery is found to account for from 60 to 80 per cent of infectious diarrhea.

Infection with Flexner and Sonne types of bacilli is world-wide in distribution and these types appear to be the most frequent causes of disease. Flexner bacilli somewhat exceed the Sonne type in prevalence. The Shiga bacillus is particularly frequent in the Orient and Egypt, but is infrequent or absent from western countries. In the United States, Flexner, Sonne and Newcastle bacilli are responsible for over 60 per cent of bacillary dysentery. The other types are infrequently reported. Dysentery bacteria are widely distributed in the population, so that healthy carriers as well as acutely infected patients constitute important sources of infection. Convalescent carriers may disseminate organisms for several weeks following an acute attack, and cultural surveys have indicated that the carrier state is usually temporary. Approximately 2.0 to 4.0 per cent of the population may harbor dysentery bacilli.

Dysentery is particularly prevalent during the summer in temperate climates and is favored by poor sanitation and lack of personal hygiene, particularly when such conditions prevail in asylums, orphanages and similar institutions. Institutional epidemic and endemic dysentery is a serious problem.

Dysentery organisms are transmitted through personal contact and by water and particularly food contaminated with fecal discharges of infected persons. Flies are at times incriminated, but they play a minor role. Epidemics are most often traceable to contaminated food and are particularly related to interrupted sanitation. Control of bacillary dysentery is thus a matter of personal hygiene and food, water and milk sanitation (see Chapter 43). Carriers among food handlers are of public health importance.

**Immunity.** Immunity in bacillary dysentery does not provide adequate protection against infection. Repeated attacks of dysentery are frequently observed.



vaccination of man is unsatisfactory, although adequate protection is conferred against experimental infections in mice. Specific agglutinins appear in blood during infection and have diagnostic value. However, significant titers are not observed in normal sera so that the results of agglutination tests must be cautiously interpreted.

**Chemotherapy and Antibiotic Therapy.** The dysentery bacteria are susceptible to drugs of the sulfonamide series, sulfadiazine, sulfathiazole and to some extent sulfaguanidine and sulfasuccidine being effective in control of the disease and carrier state. Penicillin is not effective against the dysentery group. Organisms are moderately susceptible to streptomycin *in vitro*, but the value of this antibiotic in treatment of dysentery is not yet established. Chloromycetin is of value.

## PROTEUS

Members of the genus *Proteus* are highly pleomorphic, motile, gram-negative bacteria, which characteristically produce a film-like spreading growth, liquefy gelatin and ferment glucose and sucrose, but not lactose. Organisms of this group are widely distributed in soil, manure and water and are commonly present in the intestinal contents of man and animals. In addition, members of the group are at times pathogenic. The common species is *Proteus vulgaris*.

**Morphology.** The organisms morphologically are slender rods, arranged singly or in pairs, although cocco-bacilli and longer filamentous rods may be observed. They are gram-negative and actively motile by peritrichous flagella.

The colonies of *Proteus* are particularly interesting in that they are not discrete colonies, but are typically spread in a film over the surface of solid media. This "swarming" film-like growth results from migration of the actively motile bacteria toward the border of the colony; all stages from a small film at the center of the colony to complete coverage of the surface of the dish may be observed. The growth is gray, translucent and smooth. Nonmotile organisms do not exhibit swarming, and spreading growth by motile cultures may be inhibited by the addition of 0.01 per cent sodium azide or 10 per cent of 95 per cent ethyl alcohol in the medium. Prevention of film-like growth permits isolation of other bacteria from mixed cultures containing *Proteus*.

**Metabolism.** *Proteus* cultures grow well on ordinary media at either body or room temperature and will develop either aerobically or anaerobically. They are readily destroyed by heat and disinfectants. Strains of *Proteus* vary in their nutritional requirements, some being able to use ammonium salts, others requiring amino-acid nitrogen; they generally require nicotinic acid. Dextrose and sucrose are fermented with production of acid and gas; gelatin is liquefied and indole is formed by most strains; milk is made alkaline and peptonized and  $H_2S$  is produced. Cultures of *Proteus* have an ammoniacal odor.

**Immunology.** The antigens of *Proteus* have received particular attention because of the agglutination of certain strains known as *Proteus X* by the blood

of patients having rickettsial infections. Motile strains of *Proteus* contain somatic (O) and flagellar (H) antigens, whereas nonmotile strains have only flagellar antigens. The agglutination by serum from patients having rickettsial infection (known as the **Weil-Felix reaction**) is the result of the presence of a common O antigenic component in the Rickettsiae and *Proteus X* strains. Several groups of *Proteus* are recognized by means of O agglutination; the X strains are distinguished antigenically from *Proteus vulgaris*.

**Pathogenicity.** *Proteus* is causally related to purulent infections in various locations in the body, the most important of which is the infection of the urinary bladder. Infections with pure cultures and mixtures with other bacteria may be present in wounds, the ear, the skin and deeper tissues. In the skin it may be associated with other bacteria in gangrene. In addition, *Proteus* may be cultured in large numbers from the stools in certain cases of food poisoning. However, it may also be cultured during the healing stages of other diarrheal diseases from the normal stool, so that its presence in food poisoning is not proof of causal relationship. Abscesses are usually produced in experimental animals.

***Proteus Morgani*.** The organism known as Morgan's bacillus was isolated from stools of patients having summer diarrhea. The organism, which has properties of both the coliform and *Proteus* groups, is motile, ferments dextrose but not lactose, produces indole and  $H_2S$  but does not liquefy gelatin. Milk is made alkaline. Morgan's bacillus has been associated with outbreaks of summer diarrhea in infants and has been isolated from illnesses resembling paratyphoid fever. The organism is a parasite, but its significance as a human pathogen remains in doubt.

## PSEUDOMONAS

*Pseudomonas* is the name of a group of gram-negative, motile, aerobic bacteria which generally produce soluble yellow, blue or green pigment, which may be fluorescent. Members of the genus have been recognized since the early days of bacteriology, and most species, the representative of which is *Pseudomonas fluorescens*, are common soil and water bacteria. A few species are pathogenic for animals, but the only one of medical importance is *Pseudomonas aeruginosa* (*Bacillus pyocyaneus*, *Ps. pyocyanea*). This organism was described in 1882 by Gissard from "blue pus" of infected wounds. It is noteworthy that cultures of *Pseudomonas* were early observed (1887) to be inhibitory to certain other bacteria.

**Morphology.** The members of the genus are slender motile rods of variable length, arranged singly, in pairs, short chains or clusters. Colonies are large, smooth and somewhat spreading with entire or irregular margins. The colonies are not pigmented, but under suitable conditions the medium becomes colored by diffusion of pigment.

**Metabolism.** Cultures develop on all ordinary media under aerobic conditions, but growth is poor in the absence of oxygen. *Pseudomonas aeruginosa* grows best at body temperature, whereas *Ps. fluorescens* develops more abundantly at lower temperatures.



ly at room temperature. Pigment production is best observed in dextrose-rose agar at room temperature. Cultures of *Ps. aeruginosa* ferment only rose with production of acid; gelatin is liquefied; litmus milk is peptonized; indole is produced. *Pseudomonas fluorescens* differs culturally from *Ps. aeruginosa* by a lower temperature of growth and in the failure of some strains to liquefy gelatin. *Pseudomonas* will develop in simple synthetic media without the addition of growth factors or vitamins. Citrate, lactate or glucose may be used as sources of carbon and ammonium salts provide adequate nitrogen.

**Pigment Production.** The bluish green pigment characteristic of *Ps. aeruginosa* has been found to consist of two pigments; the one, **pyocyanin**, is blue in color and may be extracted from cultures by chloroform. Pyocyanin is produced only by *Ps. aeruginosa*, is chemically a complex ring structure, which has antibiotic properties, and is reversibly oxidized and reduced. The second pigment, **fluorescin**, is a yellowish green, water-soluble dye produced by both *Ps. aeruginosa* and *Ps. fluorescens*.

**Pathogenicity.** *Pseudomonas aeruginosa* may be cultured from the skin and intestinal contents in the absence of disease. The organism is most commonly encountered in wound infections, and in mixed culture with streptococci, staphylococci and anaerobic bacteria it may be causally related to spreading cutaneous pyoderma. The organism has also been associated with ear infections and abscesses at many locations and occasionally it may be isolated in pure culture from the blood in endocarditis or from other deep tissues. In laboratory animals large inoculations of living cultures are highly fatal and small doses produce abscesses. With the exception of aureomycin and chloromycetin, antibiotics and chemotherapeutic agents are relatively ineffective against these organisms.

## THE SPIRAL BACTERIA— CHOLERA

The causative agent of cholera (*Vibrio cholerae* or *Vibrio comma*) isolated by Robert Koch in 1883. It and related forms are the only spiral bacteria of medical importance, although other members of the group are well known. *Vibrio metchnikovii* is pathogenic for birds, and spiral organisms have been associated with diseases in lower mammals and fish. The spiral bacteria are, however, typically soil and water bacteria. Four genera are commonly recognized: *Vibrio* consisting of motile, comma-shaped bacteria; *Spirillum* containing non-motile, spiral organisms; and two genera of cellulose-digesting organisms.

### THE VIBRIOS

The vibrios are short (1 to 5  $\mu$  in length), gram-negative rods, which individually are comma-shaped. The cells may, however, be arranged in circular or S-shaped pairs and spirals and bizarre pleomorphism is seen in older cultures. The bacteria are motile by means of a single polar flagellum. Cells stain readily with ordinary aniline dyes.

The colonies are 1 to 2 mm. in diameter and are not distinctive; the growth is grayish, entire, smooth, finely granular and relatively transparent. Some strains are hemolytic.

Variation of the S  $\rightarrow$  R type occurs readily and is associated with changes in cellular and colonial morphology and in antigenicity. At times a furrowed, wrinkled or rugose variant is produced.

**Physiology.** The vibrios are aerobic organisms, little or no growth being produced under anaerobic conditions. They develop best at an alkaline pH, and that media are usually adjusted to pH 7.8 to 8.0, although selective media may be adjusted to pH 9.0. Growth occurs at either room or body temperature. The vibrios are rapidly destroyed by heat, drying, chemical disinfectants and acids but are highly resistant to alkali.

Cultures grow well on ordinary laboratory media, including peptone water (1 per cent peptone in saline). The ability to grow in highly alkaline media, inhibitory to most bacteria, has been utilized in the development of selective media for the isolation of *V. comma*. Stool cultures in peptone water, pH 8.0



show an abundant growth within a few hours, and the solid medium of adonné (an alkaline blood agar medium) is widely used for the isolation of colonies.

The vibrios ferment numerous carbohydrates with production of acid, and may be classified into types on the basis of fermentation reactions. The reactions are, however, variable and do not suffice for identification. *Vibrio comma* typically ferments dextrose, galactose, maltose, mannose, sucrose and nitrol, but not arabinose, lactose or inulin. The organisms often produce gas and ammonia and liquefy gelatin and coagulated serum. The ability to reduce simultaneously nitrite from nitrate and indole from peptone in nitrate-indole medium may be detected by adding sulfuric acid to the culture. The reaction, known as the **cholera-red reaction**, depends upon the formation of a red nitroso-indole dye and is produced by *V. comma* and a number of other vibrios which produce both nitrites and indole.

The nutritive requirements of the vibrios have received some study. The cholera vibrio may be grown in a relatively simple peptone medium without added growth factors, and a number of species develop in synthetic media containing one amino acid (asparagine) or inorganic nitrogen salts as a source of nitrogen, and lactate, pyruvate or glucose, as a source of carbon.

**Chemistry.** Both proteins and carbohydrates have been identified from *V. comma*. The carbohydrates, several of which have been described, appear to be complex substances composed of simple sugars and sugar acids. The differences in chemical structure, however, do not appear to be immunologically important.

## CHOLERA

Cholera is an intestinal infection accompanied by severe toxic symptoms. It is in so far as is known is naturally limited to man. The acute diarrheal phase of cholera begins after an incubation period of 1 to 4 days and is characterized by vomiting, fever and a profuse diarrhea with loss of a large volume of fluid in typical "rice-water" stools. Within a few hours dehydration, shock and collapse supervene. Recovery may be complete within a week or complications, particularly nephritis, and death may terminate the disease in 20 to 60 per cent of cases.

The causation of cholera by *V. comma* is proved by the constant association of the vibrio with cases of the disease, by accidental and experimental human infections and less conclusively by development of antibodies following the infection. The organisms do not invade the tissues deeply, but are present within the intestinal wall and contents in exceptionally large numbers. They may also be demonstrated by direct smear of the stool. At autopsy the intestinal wall is highly edematous and the epithelial lining is severely injured and ulcerated. The systemic changes are related to intoxication and fluid loss, and include extreme leukopenia, concentration of the blood, electrolyte imbalance and nephritis. *Vibrio comma* is pathogenic for the guinea pig and less so for mice by intraperitoneal

inoculation. Infection by the intestinal route is accomplished with difficulty in immune animals, the **Pfeiffer phenomenon** (bacteriolysis of the vibrios), may be demonstrated by intraperitoneal inoculation of guinea pigs.

**Toxin.** There is no evidence that *V. comma* produces a true exotoxin. However, filtrates of old cultures and chemical extracts of the cells contain endotoxin which is heat-stable, antigenic and toxic for laboratory animals. Available evidence suggests that it may be phospholipid.

**Epidemiology.** Cholera has been recognized for centuries, but except for occasional epidemics it has been largely restricted to Asia; hence the name Asiatic or Indian cholera. During the nineteenth century, however, cholera was widespread throughout the world in a series of devastating pandemics, the first of which began in 1817 and the last of which was concluded about 1910. It was during one of these pandemics that John Snow (1854) first demonstrated the transmission of the disease through water by his studies of the Broad Street Pump epidemic in London. Since the end of the great pandemics cholera has only occasionally been present in Europe and it has been absent from the American continents. The disease is, however, endemic in China and India where more or less extensive epidemics occur annually. Geographically, cholera is spread along the trade routes of the world and epidemics have been clearly related to human travel. Epidemics also may be related to the concentration of pollution in water during dry seasons or pollution washed into streams at the beginning of the rainy season.

Cholera is acquired by ingesting contaminated food or water, and outbreaks tend to be explosive in character. The sources of infection are cases of the disease and infected healthy persons. It has been estimated that during an epidemic as many as 10 per cent of apparently healthy persons may be infected. Infection appears to be temporary, and chronic carriers, convalescent or otherwise, have not been recognized with certainty. Cholera thus exists where sanitation is poor and environmental sanitation, personal hygienic practices, isolation and quarantine are clearly important measures of control.

**Diagnosis of Cholera.** Bacteriological diagnosis of cholera depends upon demonstration of *V. comma*. Direct smears of stools during the acute stage frequently reveal vibrios. Pure cultures may be isolated directly on Dieudonné or other selective medium or from enrichment cultures in peptone water. Isolated cultures should be identified by agglutination in specific antiserum and by cultural studies, including hemolysin tests, fermentations and the cholera-reactive reaction.

**Immunity.** The cholera vibrio is motile and hence possesses both somatic (O) and flagellar (H) antigens; these antigens are analogous to those of the *Salmonella* group. Study of the O antigens of *V. comma* and related forms by the agglutination test has resulted in the recognition of several immunologic groups. However, virulent cholera vibrios are included in one group (Group I) together with certain of the relatively avirulent El Tor vibrios.

Recovery from cholera is accompanied by the presence of antibacteri-



unity. Of particular interest is the bacteriolysis of *V. comma* following peritoneal inoculation into actively or passively immunized animals (the Jer phenomenon). The most commonly used *in vitro* immune reaction is agglutination test. Vaccination against cholera appears to have value in the prevention of disease, although the immunity is incomplete. Vaccination consists

two weekly injections of a killed, relatively heavy suspension of cholera vibrio in saline. Chemotherapy is unsatisfactory.

**Other Vibrios.** Several vibrios associated with cholera-like disease in man, but generally less virulent than *V. comma*, have been described. Two of these, the El Tor and Celebes vibrios, must be distinguished from the cholera vibrio. Both the El Tor and Celebes varieties are hemolytic, whereas *V. comma* is nonhemolytic.

An anaerobic organism, *V. parvum*, has been described in association with mixed infections, such as dental pyorrhea

and other putrid infections of the mouth. The importance of this and other spirilla of the body flora is not known.

*Vibrio metchnikovii* is associated with epidemic disease in fowl and is virulent for guinea pigs. A number of other animal pathogens have been described. Furthermore, many species of vibrios have been isolated from water and some from human and animal feces. These organisms are not known to be pathogenic.



Fig. 164. Anaerobic spirillum. Smear from single colony from an anaerobic blood agar plate. Note the curved and spiral forms. (Magnification approximately  $\times 1,400$ .)

## THE SPIRILLA

The Spirilla are saprophytic organisms, the best known of which is *Rhodospirillum rubrum*. The Spirilla are usually motile, aerobic, gram-positive organisms, recognizable by their spiral morphology. *Rhodospirillum rubrum* is a large, motile organism which grows well on ordinary media. Growth is optimum at room temperature but may not occur at  $37^{\circ}\text{C}$ . Young cultures in agar are smooth, colorless and almost colorless, whereas older cultures have a pink to red tinge. The organism is relatively inactive biochemically and is nonpathogenic.

## THE GENUS HEMOPHILUS

The genus *Hemophilus* is composed of parasitic, gram-negative, nonmotile, rod-shaped bacteria which typically require enriched media for growth. The name *Hemophilus* is derived from the early observation that these organisms require or grow best in the presence of blood or hemoglobin. The first member of the genus to be described was *Hemophilus influenzae* (Pfeiffer's bacillus).



Fig. 165. *Hemophilus pertussis*. (Magnification approximately  $\times 1,600$ .) (Kral.)

(Koch-Weeks Bacillus) isolated in 1883 and associated by Pfeiffer (1892) with the disease influenza. *Hemophilus ducreyi*, named after Ducrey, who in 1890 isolated the organism from soft chancre. In 1906 Bordet and Gengou associated *Hemophilus pertussis* with whooping cough. In addition to these highly important human pathogens several other species parasitic for man and animals are recognized.

**Morphology.** The bacteria of the genus are all small ( $0.2$  to  $0.3 \mu$  in width by  $0.5$  to  $2.0 \mu$  in length), nonmotile, gram-negative rods. Pleomorphism is common; that is, longer, at times pseudo-filamentous rods may be observed, particularly in smears of infected body fluids; bipolar staining is not uncommon. On enriched media typical colonies develop in 1 to 4 days. The colonies are small, entire, transparent and homogeneous on chocolate or blood agar. Some species, such as *H. ducreyi*, are hemolytic although usually no change is produced

in blood. *Hemophilus pertussis* after some days' growth on blood-potato-glycerol agar (Bordet-Gengou medium) produces small droplet-like colonies which have a metallic sheen. Colonies of *H. influenzae* in close proximity to colonies of staphylococci or certain other bacteria are larger than those located at a distance from this second organism; this enhanced development, known as the **satellite phenomenon**, has been related to the production of vitamins by the *Staphylococcus* which are utilized by *H. influenzae*.



**Physiology.** *Hemophilus* cultures are readily destroyed by heating at 55°C for 30 minutes, by drying and by disinfectants. The organisms develop aerobically or anaerobically at temperatures near body temperature. They are inert metabolically in that they are only weakly fermentative and use few other substances. Indole and nitrite may be produced from suitable substrates. Hemolysis is variable.

The most interesting feature of the metabolism of this group of bacteria is their vitamin requirement. As indicated above, it was early noted that freshly isolated strains, and in most species all strains, failed to develop on laboratory media in the absence of blood or other body fluids. It was also observed that certain species, notably *H. influenzae*, required a second factor for growth, which was found to some extent in blood and other animal tissues and which was produced particularly by certain other bacteria, yeasts and higher plants. Further investigations have revealed that the first factor, known as the **X factor**, is associated with blood pigment and may be replaced by hemin, and that the second factor is the coenzyme di- or triphosphopyridine nucleotide. Both the X and V factors are required by *H. influenzae*, and both may be replaced by heated preparations of the enzyme catalase. Certain components of coenzyme (composed of adenine + pentose + phosphoric acid + nicotinamide), notably nicotinamide nucleoside (nicotinamide + pentose), may replace the V factor. It is assumed that these factors function in the respiration of the cell. Growth of strains of *H. pertussis*, which in early work was found to require blood for primary isolation, may occur on ordinary nutrient medium. The *parainfluenzae* require only the V factor for growth. *Hemophilus ducreyi* requires neither X nor the V factor.

**Variation.** Cultures of *Hemophilus* undergo smooth to rough ( $S \rightarrow R$ ) variation. In the case of *H. influenzae* both smooth and rough forms may be isolated from man, although the former appears to be virulent for man and animals. Freshly isolated cultures of virulent *H. pertussis* are usually smooth. The smooth form of *H. influenzae* is encapsulated, and it may be divided into immunological types, A and B. Smooth to rough variation of *H. pertussis* and *H. influenzae* occurs readily on artificial media and, although smooth cultures of the latter organism may be isolated from pathogenic sources, freshly isolated strains of *H. influenzae* frequently are rough.

**Pathogenicity and Toxin Production.** Exotoxin production by species of *Hemophilus* has not been demonstrated, although the bacterial cells and culture filtrates in large doses may be toxic to animals. The injection of large doses of virulent cultures, bacterial cells or culture filtrates is followed by development of similar pathological changes and death in suitable laboratory animals. The most characteristic change is the presence of hemorrhages in many tissues, including the adrenal glands. The living cultures are only slightly invasive. In man, species of this genus have been associated with influenza, conjunctivitis, whooping cough, soft chancre and numerous other infections, including meningitis, pneumonia, endocarditis, and upper respiratory infections.

**HEMOPHILUS INFLUENZAE**

In 1883 Koch and later Weeks associated the organism known as the Weeks bacillus with epidemic conjunctivitis, or pink eye. During the pandemic of 1892-93 Pfeiffer isolated a similar organism, which he considered to be the cause of influenza, and named it the influenza bacillus. The evidence indicates that these organisms are identical and they are, therefore, considered together as *Hemophilus influenzae*.

The frequency of isolation of *H. influenzae* from normal individuals suggests that its normal habitat is the upper respiratory tract. In this location it is commonly associated with acute infections, sinusitis, otitis media and similar diseases. Transference of the bacteria appears to be by respiratory droplets and by direct infection, as by contaminated hands, towels and handkerchiefs. *Hemophilus influenzae* is not infrequently isolated from lungs affected with bronchial pneumonia, particularly that occurring secondary to the virus disease influenza; occasionally it is the cause of endocarditis.

**Influenza.** *Hemophilus influenza* was, until the pandemic of 1918, generally considered to be the causative agent of influenza. However, extensive studies have revealed inconstant association of the bacterium with cases of the disease, the presence of the organism in the normal respiratory passages and in association with other respiratory infections. Although *H. influenzae* may be present as a secondary invader and, in swine, the animal strain *H. influenzae suis* is known to increase the severity of swine influenza, human influenza has been shown to be a virus disease.

**Conjunctivitis.** Epidemic conjunctivitis due to the Koch-Weeks bacillus is recognized in many parts of the world. The disease is highly contagious, affects chiefly children and is usually self-limiting.

**Meningitis.** Influenzal meningitis is caused by *H. influenzae* and bears a close relationship to the virus disease influenza. The disease is highly fatal (over 90 per cent mortality in the absence of specific treatment) and occurs in children, usually less than two years of age, in whom *H. influenzae* is among the most important causes of meningitis. Pure cultures of *H. influenzae* are isolated from the cerebrospinal fluid in cases of meningitis due to this organism. The primary focus of infection appears to be the respiratory passages, often in the presence of otitis media.

**Antigenic Structure of Hemophilus Influenzae.** The antigenic structure of strains of *H. influenzae* is incompletely known. However, a cell-associated protein, which is antigenic, appears to be common to most strains. Other cell-associated antigens appear to be heterogeneous, and agglutination tests, particularly with rough forms, are often strain specific. On the other hand, recent work with smooth cultures has revealed that these bacteria are encapsulated and that they may be classified into specific types by agglutination, precipitation and capsular swelling reactions. The type-specific antigen has been demonstrated to be a polysaccharide and presumably is associated with the capsule. The relation



ulence is indicated by the value of type-specific antiserum in treatment of infections due to *H. influenzae*. A total of six types, Types A, B, C, D, E and F, has been recognized and the polysaccharides of Types A, B, C, D and E have been studied. Types A and B are best known, and the latter is particularly associated with human infections. The influenza bacillus is antigenically related to the Koch-Weeks bacillus and to certain pneumococcal types.

**Treatment of Hemophilus Influenzae Infections.** Influenzal meningitis, from the point of view of frequency and mortality is the most important infection caused by *H. influenzae*, has presented until recently a particularly difficult therapeutic problem. Within the last ten years, however, specific antisera, notably Alexander's Type B specific rabbit immune serum, sulfonamide drugs, aureomycin and streptomycin have proved of value in the treatment of infections caused by *H. influenzae*. Of the sulfonamide drugs, sulfapyridine and particularly sulfadiazine either alone or in combination with immune serum are effective. The therapy of choice at the present time is, however, streptomycin.

## HEMOPHILUS PERTUSSIS

### *Whooping Cough*

Whooping cough (pertussis) is second in importance only to measles among contagious diseases of children. The causative rôle of *Hemophilus pertussis* (Bordet-Gengou Bacillus) is shown by its constant presence in diseased, and its absence in normal, individuals, by reproduction of whooping cough in man and by production of a similar disease in animals by inoculation of *H. pertussis* and by the specific protection afforded against the disease by vaccines of *H. pertussis*. Whooping cough is an acute infection of the respiratory passages, the trachea, bronchi and at times the alveoli (air spaces) of the lung. The characteristic symptom of the disease is the severe paroxysmal coughing. During the acute stage of the disease *H. pertussis* is present in large numbers upon and between the cilia of the respiratory epithelium and in the tenacious, sticky mucous secretion. The trachea and bronchi are the site of acute inflammation, which is responsible for the characteristic coughing. There is a general lymphocytic infiltration of the tissue. However, the bacteria remain localized in the respiratory passages.

**Epidemiology.** Whooping cough is transmitted from person to person by direct infection, particularly by infected droplets from the nose and throat. Infants are most infectious during the early stages when the disease may be unrecognized. There are approximately 180,000 cases of whooping cough reported annually in the United States, most of which occur in children. Epidemics occur periodically, although the disease is continuously present in the large cities. The disease is more prevalent during the winter months of the year. Deaths are relatively few considering the high morbidity, the death rate being variable but approximately 2.0 per 100,000 population. However, whooping cough and associated pneumonia are important causes of death among infants less than one year of age, in whom the case fatality may be as high as 50 per cent.

The infants of immune mothers are protected during the early months of congenital passive immunity. Although whooping cough affects children rarely, nonimmune adults may acquire the disease and are often severely affected.

**Diagnosis.** The bacteriological diagnosis of whooping cough is made by demonstrating typical *H. pertussis* on cough plates of Bordet-Gengou. The plates are inoculated by holding them a few inches from the mouth and nose of the patient during coughing, are incubated at 37° C for 3 to 5 days and are examined for the presence of typical metallic colonies. Cough plates are usually strongly positive during acute stages of the disease. Serological tests are of little or no value in diagnosis, although the serum of patients convalescing from whooping cough usually contains complement-fixing antibodies against *H. pertussis* antigen.

**Antigenic Types.** Freshly isolated strains of *H. pertussis* appear smooth and to belong to a single antigenic type. Strains which have been cultivated in the laboratory, however, differ antigenically from freshly isolated virulent cultures and are regarded as being in the rough or intermediate state. Smooth to rough variation, with associated changes in antigenicity and virulence, occurs readily. Strains of *H. pertussis* may be divided into four immunological types, designated Phase I, II, III and IV, respectively, which correspond to the fully virulent smooth state, the intermediate forms and the rough avirulent cultures. It is important that only fully virulent Phase I cultures are antigenically suitable for protective immunization.

*Hemophilus pertussis* is antigenically related to the para-pertussis bacterium and to *Brucella bronchiseptica*. It is interesting that a polysaccharide fraction of *Br. bronchiseptica* is reported to give protection in mice against infection with *H. pertussis*.

**Toxin Production.** Extracts of *H. pertussis* and filtrates of cultures are highly toxic and necrotizing in animals and are antigenic. Furthermore, treatment of toxin with formalin results in formation of toxoid which has immunological value. The toxicity of the cellular substances and that of the filtrates of cultures appear to be identical and are similar in many of their properties to the endotoxins of other bacteria.

**Immunity.** One attack of whooping cough provides a high degree of resistance against subsequent infection which is associated with the presence of specific immune bodies. Furthermore, protection against infection is obtained by artificial immunization in a high proportion of cases. Although formalin has received some trial, the material most commonly used for immunization is a vaccine composed of killed *H. pertussis* Phase I. In the United States vaccination is usually performed by giving multiple injections at weekly intervals. The Sauer vaccine containing 10 or 20 billion organisms per milliliter. Large doses are required to provide adequate protection. The presence of immunity following vaccination is correlated with the presence of complement-fixing antibodies. Recently it has been associated with a positive skin reaction to *H. pertussis* antigen.

Until recently no satisfactory antiserum against *H. pertussis* has been available.



for clinical use. However, human immune serum has now been obtained from adults vaccinated with *H. pertussis* vaccine. Both liquid and lyophilized sera have now been prepared and appear to have value in preventing whooping cough in exposed individuals. However, serum is of no value in treatment of the disease. It is interesting that vaccination of expectant mothers has been tried with some indication of success for the prevention of whooping cough in newborn infants by congenital passive immunity. The last development is in accord with resistance against whooping cough of infants of naturally immune mothers. *Hemophilus pertussis* is sensitive to streptomycin, and this antibiotic has been used successfully in the treatment of experimental infections in mice. Aureomycin is also active against *H. pertussis*.

## HEMOPHILUS DUCREYI

### *Chancroid*

*Hemophilus ducreyi* is the causative agent of the venereal disease, soft chancre or chancroid. The disease is limited to a soft ulceration of the genitalia, usually with associated enlargement of the regional lymph nodes. The presence of a general immunological response is, however, indicated by the development of sensitivity to the intracutaneous inoculation of killed bacteria. There is little protective immunity and multiple lesions or auto-inoculation may occur. Transmission is by personal contact, usually sexual.

*Hemophilus ducreyi* may be identified by cultures of the purulent exudate from the lesion, and it may be seen in smears of the same material, although these are not clearly diagnostic. Diagnosis may be aided by the presence of a positive skin test, which is manifest by the development of an area of redness and induration within 48 hours following the intracutaneous injection of a suspension of killed *H. ducreyi*.

Treatment of chancroid may be local or general and includes use of chemotherapy and antibiotic therapy. Unlike other pathogenic members of the group, *H. ducreyi* is reported to be sensitive to penicillin and to drugs of the sulfonamide series.

## SCCELLANEOUS BACTERIA OF THE GENUS HEMOPHILUS

*Hemophilus duplex* or the Morax-Axenfeld bacillus has been associated with conjunctivitis in man. The organism requires enriched media for growth and is nonpathogenic for animals. The conjunctivitis yields readily to proper chemotherapy.

*Hemophilus parainfluenzae* differs immunologically and in growth requirements from *H. influenzae*.

*Hemophilus para-pertussis* has been isolated from patients having a disease very resembling whooping cough. Indeed this organism is distinguished with difficulty from *H. pertussis*, but it differs culturally and immunologically from the latter species.

## THE GENUS BRUCELLA— BRUCELLOSIS, UNDULANT FEVER

The first species of the genus to be identified was *Brucella melitensis*, which was isolated by Bruce in 1887 from patients with Malta or Mediterranean fever. In 1897 Bang isolated *Bacillus abortus* (Bang) and established the causal relationship of this organism to infectious abortion of cattle. The third member



Fig. 166. Photomicrograph of *Brucella melitensis*. (Magnification approximately  $\times 1,600$ .) (Kral.)

of the group, *Brucella suis*, was isolated in 1914 from swine. Although undulant fever in man and Bang's disease (infectious abortion) of animals had been recognized in many parts of the world, the relationship between the causative organisms of these diseases was not established until 1918 by Evans, and the name *Brucella* was given to the group in 1920 by Meyer and Shaw. The organisms are now properly named *Brucella melitensis*, *Brucella abortus* and *Brucella suis*, and the disease in both man and animals is commonly called brucellosis.

**Morphology and Staining.** *Brucella melitensis* typically appears as a coccobacillus arranged singly or in pairs, whereas *Br. abortus* and *Br. suis* are more bacillary in form. All species are small, approximately  $0.5 \mu$  in width and  $1-3 \mu$  in length, nonmotile, gram-negative rods; capsules may be seen on organisms from virulent smooth cultures. The colonies are translucent, amorphous, smooth and convex.

Smooth to rough variation occurs readily on artificial media and is associated with a loss of virulence and with altered agglutinability.

**Physiology.** The *Brucellae* are aerobic organisms. However, *Br. abortus* requires increased  $\text{CO}_2$  tension (10 per cent) for isolation and growth of fresh isolated cultures, and the growth of *Br. suis* is favored by a similar  $\text{CO}_2$  tension. The optimum pH for growth is 6.6 to 6.8, and development is best at  $37^\circ\text{C}$ .



organisms do not liquefy gelatin; glucose is utilized to some extent by all; milk is made alkaline. The species differ in the quantitative production of hydrogen sulfide and ammonia.

The *Brucellae* survive many days in soil, water, milk and milk products. They are, however, readily killed by pasteurizing temperatures and sunlight, and are usually resistant to disinfectants.

Species of *Brucella* may be differentiated by their susceptibilities to the static action of dyes. *Brucella melitensis* is able to grow on suitable agar containing 1:50,000 aqueous thionin and 1:25,000 basic fuchsin, whereas *Br. abortus* is inhibited by thionin and *Br. suis* is unable to grow on media containing basic fuchsin.

The *Brucellae* grow well on liquid or solid liver infusion and bacto-tryptose media. Although cultures may develop in initial transplants in synthetic amino acid media, continued growth is dependent upon the presence of accessory growth factors. Cultures of *Brucella* differ in their vitamin requirements, although for more of the following are required or are stimulatory for most strains: nicotinic acid, nicotinamide, pantothenic acid and biotin.

**Antigens of *Brucella*.** The species of *Brucella* are closely related antigenically. Two heat-stable antigens, designated A and M, which are present in different proportions in each of the species, have been described. *Brucella melitensis* may be differentiated by the large proportion of M antigen, whereas *Br. suis* and *Br. abortus* contain a large proportion of the A substance. The antigenic differences, which are, thus, quantitative, may be demonstrated by the precipitation and agglutinin-absorption tests.

**Epidemiology.** Human brucellosis is in almost all instances secondary to the disease in animals and is acquired through contact with infected animals, milk and milk products. Infection is rarely, if ever, transmitted from man to man. It is readily acquired by personnel working with *Brucella* organisms. The disease occurs in many areas of the world and may be caused by any of the species of *Brucella*. The relative prevalence of human infection with the different varieties tends to reflect the degree of contact with the specific lower animals. Thus, in areas such as the Mediterranean region, where goats and sheep are commonly raised, the caprine *Br. melitensis* is predominant; and in regions dependent upon cattle and swine *Br. abortus* and *Br. suis* are responsible for the majority of human infections.

The incidence of brucellosis in man is relatively poorly known, because of its insidious nature in many cases and the protean manifestations with attendant difficulties of diagnosis. The several thousand cases reported annually in the United States probably represent only a fraction of the total number of infections. Available evidence suggests that brucellosis is more prevalent in rural populations than among agricultural and packing-house workers than in urban areas or in the general population. The disease affects all age groups, but it is more frequent in males than in females.

Many species of animals may acquire infection with *Brucella*. However, the

prevalence is greatest in cattle, hogs, goats and sheep. Cattle most often *Br. abortus* but are also susceptible to *Br. suis* and *Br. melitensis*. Swine infected with *Br. suis* and *Br. melitensis* but appear to be resistant to *Br. a*. Goats and sheep are the primary reservoirs of *Br. melitensis*. Infection is transferred from one to another species, a fact which indicates the danger allowing the several species to graze in the same pasture. *Brucella* may be present in the tissues, the milk, urine, semen and discharges (e.g., uterine discharges) of infected animals. Animals may acquire infection by contact or the ingestion of contaminated food. Invasion may occur through the skin or the mucous membranes of the conjunctivae and intestinal and genital tracts. Introduction of an infected animal into a herd is soon followed by spread of the disease to the other, usually uninfected stock.

**Pathogenesis.** The most prominent symptom of acute brucellosis in domestic animals, and the one which accounts for its name of infectious abortion, is abortion of the recently infected female animal. However, abortion occurs not only in the acute disease and then only in a fraction of pregnant females. The disease is in actuality a generalized infection which affects both sexes and may be chronic or entirely asymptomatic. Cattle may develop abscesses in the tissues and infection of the joints is common. In goats the disease runs a more chronic course with general debility. Swine may remain asymptomatic. *Brucella* has been isolated from many tissues and body fluids of infected animals. Localization in the udder and in both the male and female genito-urinary tracts is particularly important to transmission of the disease in nature.

Among laboratory animals, rabbits, mice and monkeys may be infected, although the guinea pig is almost universally used for diagnostic and experimental tests. Following infection by the dermal, conjunctival or intraperitoneal routes a generalized disease is produced. Typical lesions include nodules of the spleen and spleen, enlargement of the lymphatic tissues and, in the male, orchitis and abscesses of the testes and epididymides.

The symptoms of brucellosis in man are highly varied. The incubation period may be as short as one week or as long as several months. *Brucella melitensis* and *Br. suis* infections are in general more severe than those with *Br. abortus*. Although the case fatality rate is probably very low, disability may be prolonged and relapses are frequent. The acute disease is a febrile illness, which may occur in any of several types: (1) the undulant, characterized by a wave-like temperature curve which gradually returns to normal, weakness, aching, loss of appetite, night sweats, debility and malaise; (2) the intermittent, characterized by intermittent febrile and afebrile periods, general debility, malaise, aching and often stiffness of the back and joints; (3) the ambulatory, a mild disease in general similar to the intermittent type; and (4) the malignant, a severe febrile illness with delirium and coma. The chronic disease has many manifestations. The symptoms may be nonspecific, pulmonary, rheumatoid or may be related to localization of infection in many tissues. The disease is a generalized infection and the pathological changes are those of chronic inflammation. T



ally evidence of activity of the lymphoid-macrophage system, and nodular lesions and abscesses similar to those in animals may be found in many tissues. Infection is rarely observed during the course of human brucellosis. In persons sensitive to *Brucella* antigens, an erythematous skin eruption may be observed.

**Diagnosis by Laboratory Methods.** The diagnosis of brucellosis is in part dependent upon laboratory methods. The tests of value include isolation of the causative organisms from body fluids or tissues, the agglutination reaction, the opsono-cytophagic reaction, and, although not strictly a laboratory method, the brucellergen reaction.

**Isolation.** *Brucella* may often be isolated from the blood of man during the acute disease and from the tissues, discharges and milk of infected animals. For culture is inoculated in 5- to 10 ml. amounts into tryptose broth and the culture is incubated in an atmosphere of 10 per cent  $\text{CO}_2$ . Frequent subcultures on agar media are made for the isolation of suspicious organisms. The *Brucella* is identified by agglutination in specific immune serum, growth on media containing thionin and basic fuchsin,  $\text{H}_2\text{S}$  production and  $\text{CO}_2$  requirement. *Brucella* may be isolated from infected animal tissues and, of great importance in human infection, may be cultivated from milk by suitable methods. In the case of milk the gravity cream is inoculated onto tryptose agar medium, since gravity cream contains a higher concentration of bacteria than does the whole milk.

Inoculation of guinea pigs with suspected tissues or body fluids, including the blood, followed by the demonstration of immune bodies in the serum of these animals and cultivation of the organisms from the nodular lesions of positive guinea pigs at times gives positive results when direct culture methods are unsatisfactory. Isolation of *Brucella* by either cultivation or animal inoculation is, of course, conclusive evidence of infection, and no other single test is so highly diagnostic.

**The Agglutination Reaction.** The agglutination reaction with the blood serum of man and animals suspected of infection is often useful in diagnosis. Interpretation of the test must be made with caution, however, since the agglutinins do not usually appear until ten days after onset of the acute disease and persist in the immune individual. The agglutinin titer may vary from less than 1:10 to more than 1:1,000. A titer of over 1:80 and a rise in titer during the course of the disease are probably significant. In performance of the test either the tube reaction with a heated or unheated suspension of known *Brucella* in sterilized saline or the rapid macroscopic test, using a specially prepared concentrated bacterial suspension, may be employed.

Cross-reactions may be obtained in the agglutination test with serums containing antibodies against *Pasteurella tularensis* and *Vibrio comma*.

**The Opsono-Cytophagic Reaction.** The opsono-cytophagic test measures the phagocytic activity of whole blood against *Brucella*. In the test 0.1 ml. of whole citrated blood being tested is mixed with an equal volume of a

freshly prepared, standardized suspension of *Brucella* and the mixture incubated for 30 minutes. Stained films are then prepared and the number of leucocytes showing phagocytosis and the number of bacteria per leucocyte determined by microscopic examination of the films. In the normal susceptible individual, less than 20 per cent of leucocytes show phagocytosis and each cell contains fewer than 20 organisms. Less than 40 per cent of the leucocytes show phagocytosis in the presence of infection, but each cell contains 40 or more bacteria. In immune blood 60 to 100 per cent of leucocytes show phagocytosis (40 or more bacteria per cell). The combination of a positive agglutination reaction and a low or moderate phagocytic reaction constitutes good immunological evidence of infection, whereas a positive agglutination reaction and a marked degree of phagocytosis are typical of the immune reaction.

**The Brucellergen Reaction.** The brucellergen reaction is a test for hypersensitivity to *Brucella* antigen performed by the intracutaneous injection of 0.1 ml. of a standardized solution of the protein-nucleate fraction of *B. abortus*. In a positive reaction an area of redness, edema and induration appears at the site of injection within 48 hours. A positive skin reaction has been found to occur frequently in acute and chronic brucellosis and in the immune individual. However, a positive reaction may occur in other individuals and alone is not suggestive of infection.

**Immunity and Chemotherapy.** The prevalence of positive agglutination reactions in the absence of a history of infection and the demonstration of chronic brucellosis, together with the recorded low incidence of the acute disease, indicate a relatively high resistance to the disease in man. In animals, like man, the disease tends to be nonfatal. On the other hand, prevention of the disease by vaccination and treatment of infection with immune serum have been disappointing and are considered to be unsatisfactory. Similarly, treatment of infection with streptomycin has been only partially successful, although the antibiotic activity against *Brucella* may be demonstrated. However, the combined streptomycin and sulfadiazine appears promising, as does treatment with aureomycin and chloromycetin.

**Control.** Control of brucellosis in man is dependent upon control of the disease in infected animals. The creation of *Brucella*-free herds of domestic animals by the eradication of animals that give evidence of infection, separation of infected animals from pasturage, control of sale of infected animals and regulation of dairy herds are important veterinary measures. Pasteurization of milk and other dairy products, rejection of infected animals by abattoir inspectors and adequate cooking of meat provide additional safeguards against human infection.

***Brucella Bronchiseptica.*** *Brucella bronchiseptica* is a motile bacterium related immunologically and culturally to members of the genus *Brucella* and *Hemophilus*. This organism, which in the past was thought to cause distemper, is now recognized as a parasite of the respiratory tract of many animals, particularly rodents. It is isolated frequently from the lungs and bronchial secretions in bronchopneumonia of rodents.



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## THE GENUS PASTEURELLA— PLAGUE AND TULAREMIA

The genus *Pasteurella* is composed of a group of small, parasitic, gram-negative rods which usually show bipolar staining. Spores are not formed and the organisms are not unusually resistant to deleterious agents. The organisms are strictly aerobic and possess limited powers of fermentation. Members of the genus produce disease in man and animals, the most important of which are plague and tularemia. The type species of the group is *Pasteurella avicida* (1880), the cause of fowl cholera and septicemia in domestic species of wild birds. Another member of the genus was, however, isolated in 1912 from a disease of deer. The plague bacillus (*Past. pestis*) was described separately by Yersin and Kitasato (1894), and *tularensis* was isolated in 1912 by Hensley and Chapin from wild rodents in Montana. At present a total of eight species are recognized: *Past. pestis*, *Past. tularensis*, *Past. pseudotuberculosis*, and five species which cause highly fatal hemorrhagic septicemias with hemorrhages in lower respiratory tract, and *Past. pseudotuberculosis*, a disease of guinea pigs which rarely infects man.

**Morphology.** Organisms of the group consist of small ovoid rods measuring 0.5 to 1.0 µ in length. They stain with the usual Gram dyes, are gram-negative and by the older methods exhibit bipolar staining.

*Pasteurella tularensis* and *Past. pseudotuberculosis* are the only species which are motile. Capsules are sometimes formed. Colonies are usually granular, small, raised and translucent on surface. Cultures are subject to variation of the S → R type; avirulent variants are well known.

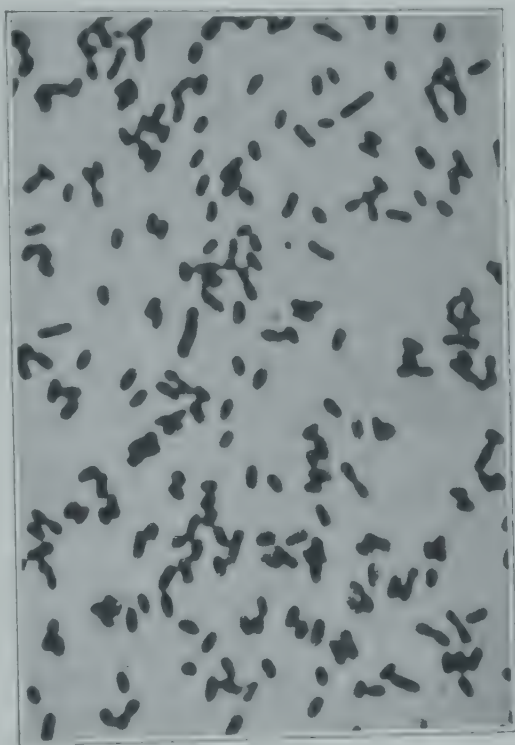


Fig. 167. *Pasteurella pestis*, the plague bacillus. (Magnification approximately  $\times 2,000$ .) (Kral.)

**Metabolism.** With the exception of *Past. tularensis*, members of the genus develop well on ordinary culture media at 37° C. The plague bacillus, when cultivated in a medium composed of amino acids, of which proline, cysteine, and phenylalanine are indispensable. Glucose and lactate were found to be the main sources of energy and, although a number of vitamins appeared to be stimulatory, none was essential for growth. On the other hand, *Past. tularensis* requires a number of growth factors. This species has been cultivated in a basic amino acid medium containing pantothenate, nicotinic and pimelic acids and liver concentrate. Recently in a casein hydrolysate medium with thiamin as the only added growth factor. For ordinary purposes this organism grows well on coagulated serum medium or blood-glucose-cystine agar.

*Pasteurella* species ferment carbohydrates without production of gas. These reactions, however, possess little differential value. The fermentations of *P. pestis* are variable; lactose and rhamnose are not attacked. *Pasteurella tularensis* ferments glucose, maltose and mannose and may attack a number of other carbohydrates. Gelatin is not liquefied, nor are other proteins usually digested.

**Pathogenicity.** *Pasteurella* species are best known by the diseases which they produce. All are primarily pathogens of lower animals and only secondarily of man. The bacteria are highly invasive and characteristically produce septicemic infections. The lymph nodes, spleen and lungs are often sites of localization and are frequently infected. Liver damage is frequent. Embolic phenomena, *i.e.*, small hemorrhages, are associated with infection in the hemorrhagic septicemias of lower animals.

Natural infection with one or another species is known to occur in domestic and wild birds, wild rodents, rabbits, swine and herbivores, including cattle, buffalo and deer. In the laboratory, guinea pigs, mice, rats and monkeys are commonly used for experimental studies.

## **PASTEURELLA PESTIS**

### *Plague*

Plague has been one of the devastating diseases of mankind since antiquity. It has spread repeatedly from its endemic foci in Asia to involve wide areas of the world in fearful pandemics that have caused a loss of life proportionate to that unsurpassed by any other epidemic disease. Plague was well known to the Greeks, Hindus, Hebrews, and Romans and during the Middle Ages swept over Europe as the terrorizing Black Death. The last widespread outbreak began in the latter nineteenth century in China, whence it spread to all major continents, including North America. Endemic plague has been recognized in the United States since 1900. The association of human disease with plague in rodents was established by Ogata (1897) and by others soon after the etiology of the disease became known (1894).

**Epidemiology.** Plague is primarily a disease of rodents which secondarily affects man, but which may then be transmitted from person to person. The rodent first recognized to be a reservoir of plague, and the one intimately



with epidemics of human plague, is the rat. Both the gray rat (*Rattus norvegicus*) and the black house rat (*Rattus rattus*) are known to harbor infection. The disease is transmitted among these animals by rat fleas and to a lesser extent by the rat louse. Murine plague may occur in highly fatal epizootics in which as many as one-fourth of examined rats are found infected and a large number of rodents succumb to the disease. When such epizootics occur, human plague is particularly liable to infection by fleas which leave the dead animals. Outbreaks of human plague are chronologically related to such epizootics, the human disease preceding by days or weeks the wave of fatalities in man. Distribution of plague to widespread geographical areas is accomplished by transport of infected rodents along the trade routes. Ships are particularly worthy carriers of rats, although trains and other vehicles may also be infected. Endemic foci of murine plague have been recognized in Asia, particularly in China, Burma and India.

Although plague in rats is the most important source of the human disease, other types of rodent infection are now clearly recognized. Investigations since 1900 have revealed plague infection in a number of forest and field rodents from widely separated geographical areas, including Asia, Russia, South Africa and the United States. This rural disease, known as **sylvatic plague**, constitutes a reservoir of infection for man, and presumably for rats, that is particularly difficult to control. In Asia, for example, outbreaks have not infrequently been reported in the rather remote interior before they have involved the more densely populated and rat-infested coastal areas. The tarabagan, a small rodent hunted for its fur, has been the source of human outbreaks in Inner Mongolia. In the United States, plague of epidemic proportions has not been traced to wild rodent infection, although sporadic human infections have been reported. However, the demonstration of plague in several varieties of squirrels, mice and field rats in the Pacific region that now includes the Pacific coast, mountain and some northern plains states has revealed a reservoir of potential danger. Although the infection may have been present in the Pacific states for some time, the evidence suggests it was introduced about 1900, became established in the city of San Francisco and in the state of Washington and has subsequently spread to its present area. In the past, epidemics of both bubonic and pneumonic plague have occurred in several seaports, and the disease has been recognized and controlled often in maritime quarantine stations.

Although several species of fleas may be infected experimentally, the most important natural vector of plague is the Asiatic rat flea, *Xenopsylla cheopis*. Fleas of other genera (*Ceratophyllus*, *Ctenopsyllus*, etc.) have been found infected and appear to be important in the transmission of sylvatic plague. Fleas ingest plague bacilli with the blood meal from an infected host. The organisms multiply in the gastro-intestinal tract of the flea and the mass of bacteria may obstruct the narrow lumen of the proventriculus and thus prevent entrance of blood into the stomach. Such fleas are unable to feed successfully, and it is now generally held that plague is transferred by regurgitation into the bite during violent

feeding efforts. An alternative method is contamination of the bite by infected fleas or with feces of the flea. The obstruction of the digestive temporary and infected fleas may live for several weeks. Fleas do not infest man but may do so in the absence of a suitable lower animal host. Infestation is more likely during epizootics when the natural rodent host is reduced in number.

Human plague is usually of the bubonic type acquired by flea transmission from rodents. At times, however, infection is transferred directly from man by infected droplets from the respiratory tract. The latter disease, as pneumonic plague, may account for large epidemics, but it usually is restricted to persons in close contact with a patient having plague.

**Pathology and Bacteriology.** Plague is a highly fatal disease in man. Necrosis, or death of tissue, hemorrhage and edema are commonly found at the site of invasion. The lymph nodes are swollen, infected and often hemorrhagic. The lungs are congested; the liver and spleen are usually enlarged and the surface may be mottled with small necrotic spots. In naturally infected animals (an enlarged, hemorrhagic lymph node which may become purulent) is commonly present. *Pasteurella pestis* is found in the blood and tissues.

Human plague is an acute febrile disease which may be present in the bubonic or pneumonic form. Buboes are present in about three-fourth of human cases. Septicemia with hemorrhages into the skin and tissues is commonly present. The pneumonic variety is characterized by pneumonia and the presence of organisms in the sputum.

The plague bacilli are widely distributed in the buboes, spleen, blood and other tissues of infected man and animals. Material for bacteriological examination may be obtained by aspiration of a bubo or it may consist of tissues, spleen or blood. In properly stained smears of infectious material the organism frequently may be seen as typical bipolar-staining coccobacilli. Animal pathogenicity tests constitute the best method of diagnosis. Usually guinea pigs are inoculated either subcutaneously or by rubbing the shaved skin of the abdomen with suspected material. Typical disease will result in a few days. Cultural identification of *Past. pestis* is largely unsatisfactory, and agglutination tests of limited value. Differentiation from tularemia may be made, however, by specific immune reactions with human or animal immune serum, and less satisfactorily by the pathology in animals. All precautions, including aseptic technique, screening and elimination of ectoparasites, must be taken to protect personnel and to prevent spread of infection in the animal room.

**Immunity and Chemotherapy.** Virulent and avirulent strains of *pestis* are similar immunologically. Infection confers a solid immunity and is stable, avirulent strains are effective immunizing agents for animals. Inactivated, heat-killed vaccines, such as the Haffkine vaccine, have some value, and live tests with vaccines of living avirulent cultures suggest the possibility of their use for the protection of man. Immune serum has not proved satisfactory for the treatment of plague.



The value of chemotherapeutic and antibiotic agents has not been established. **Control.** The control of plague is primarily a problem in the control of rats and other rodents, elimination of their contact with man by engineering and other measures, control of the ectoparasite population by insecticides, hygienic regulation of commerce and attempted control of contact with forest and field animals. These control methods are discussed in Chapter 43. In addition, isolation of patients with plague should be carried out to prevent pneumonic infection, and personal protective measures should be used by persons in endemic areas.

## PASTEURELLA TULARENSIS

### *Tularemia*

Tularemia in animals was first described in 1910 by McCoy and Chapin, in 1912 also identified *Pasteurella tularensis* as the causative agent. The first serologically proved human infection was reported two years later, although clinical descriptions were made as early as 1907. Since these early reports from the United States, tularemia has been recognized in many parts of the world as a disease of animals secondarily transmissible to man.

**Epidemiology.** Tularemia has been reported from the United States, Canada, Sweden, Russia, Central and Southeastern Europe and Japan. The infection has been recognized in many species of mammals and birds native to the geographical areas. Wild rodents, including squirrels, wild mice, chipmunks, the lemming and hamster, and lagomorphs (rabbits) are particularly important animal reservoirs. The cottontail rabbits (*Sylvilagus sp.*) on epidemiological grounds appear to be the most important and perhaps the definitive reservoir animal. Human tularemia has been reported from all states of the United States, but has been recognized most frequently in the central states of Ohio and Mississippi river valleys. Approximately 90 per cent of human cases are acquired through contact with infected rabbits (70 per cent from cottontail rabbits), and only 10 per cent result from contact with other animals from tick and deer-fly bites. Domestic rabbits are not known to be infected. Cases of tularemia have been reported during all months of the year; many encountered during the hunting season, although some tick-borne infection may be prevalent from March to August in the northern and from February to October in the southern states; and disease may be transmitted by deer flies from June to September.

It is estimated that 1 per cent or more of wild rabbits are infected with tularemia. The disease is transmitted by the ectoparasites of these and other reservoir animals. A number of arthropods have been found to be successful vectors of tularemia, the most important of which is the rabbit tick (*Haemaphysalis leporis-palustris*) which is largely responsible for the maintenance of the disease in nature. Other ticks of the genus *Derma-centor* (*D. andersoni* and *D. variabilis*) and the genus *Ixodes*, the rabbit louse, the deer fly (*Chrysops* spp.) and the horse fly are also important vectors. It is particularly interesting

that in the spotted fever tick (*D. andersoni*), *Pasteurella tularensis* is carried through the egg from one to the next generation of ticks.

Human infection is most often acquired by handling infected fresh tissues, such as occurs during preparation of the carcass for food, or ingestion of partially cooked meat or, as indicated above, the bite of an arthropod vector may also result in infection. *Pasteurella tularensis* has been demonstrated in water in sufficient numbers to infect laboratory animals, and water-borne epidemics in man and animals have been reported.

**Pathology and Bacteriology.** Rabbits and other animals infected with tularemia exhibit findings typical of the disease. The liver, spleen, lung and bone marrow are studded with small whitish lesions giving rise to the mottled appearance grossly typical of infection. These spots consist of necrotic accumulations of inflammatory cells and small caseous abscesses. Older lesions tend to be larger in size. In general the lesions resemble somewhat those of tuberculosis. Injury to small blood vessels is common. The animals are slowly and obviously ill and frequently die of the infection.

Tularemia in man is an acute febrile and debilitating disease which is fatal in about 5 per cent of the cases. Convalescence requires several months. Several types of the disease are recognized. In the most frequent, the **ulcero-glandular** type, invasion occurs through small abrasions in the skin, usually that of the hands or face. A necrotic ulcer develops locally after some days and the regional lymph nodes become painfully enlarged and may abscess. In the **oculoglandular** type invasion occurs through the conjunctiva, causing a severe conjunctivitis with associated involvement of the lymph nodes. The **pneumonic** type is characterized by the predominance of involvement of the lungs, resulting in tularemic pneumonia, and in the **intestinal** or **typhoidal** type there are no localizing signs or lymph node involvement. As in animals, the lesions are necrotic and inflammatory, the spleen, liver, lymph nodes and lungs having extensive areas of inflammation, with necrosis and caseous abscesses which sometimes resemble tubercles.

In properly stained material, *Past. tularensis* may be observed in the phagocytes and occasionally in the tissue cells of the lesions. By suitable methods it may be cultivated from the tissues and rarely from the blood. The bacteria are generally distributed throughout the body. The isolation of *Past. tularensis* should be undertaken only by highly skilled personnel, since laboratory infection is common. Although direct isolation on culture media may be successful, the more common and fruitful method is the inoculation of experimental animals, usually the rabbit or guinea pig, with infectious material, and the observation of the disease in these animals. Isolation of *Past. tularensis* is the method commonly used in experimental studies of the disease in animals and arthropod vectors. The human disease is, however, better diagnosed by immunological methods.

**Immunity.** An attack of tularemia confers a high degree of immunity. The convalescent serum of man and animals contains agglutinins (antibodies) against *Past. tularensis* in a titer of at least 1:80 and often 1:320 or higher. Agglutination



It may usually be demonstrated after the first week of the human disease, but it may persist for many months or years. Antiserums containing agglutinins against *P. tularensis* react also with bacteria of the genus *Brucella*. Two skin tests have been described in tularemia: one, a toxic reaction in rabbits to formalinized cultures of *Past. tularensis*, which is neutralized by specific antiserum; and the Foshay reaction of immediate redness and swelling (erythema and edema) at the site of injection of specific immune serum. The diagnostic value of these tests is uncertain.

Vaccines composed of suspensions of killed *Past. tularensis* have been used on a small scale for the protection of animals and man. The protection conferred by vaccination is, however, incomplete. Similarly, immune serum has produced equivocal results in the treatment of tularemia, although the Foshay tularemic goat serum has given encouraging results. At present, however, treatment of tularemia with antibiotics is the method of choice.

**Chemotherapy.** Drugs of the sulfonamide series and penicillin have been found ineffective against tularemia. On the other hand, streptomycin is bacteriostatic and bactericidal *in vitro* and treatment of human and animal infections with this antibiotic aureomycin and chloromycetin appears to be highly successful.

## MISCELLANEOUS BACTERIA

## LACTOBACILLUS

The Lactobacilli are gram-positive, nonmotile, nonsporulating rods which are found widely distributed in nature from both plant and animal sources. These bacteria, like the streptococci, characteristically ferment carbohydrates with

production of lactic acid. They are further characterized by their acid tolerance and their ability to grow in a relatively high concentration of acid, pH 5.0 or below. The medical importance of the lactobacilli is related to their frequent presence in the bacterial flora of the mouth, intestinal tract and female genital tract, their association with dental caries and cavitation and their importance in the dairy and cheese industries.

The lactobacilli vary from short coccobacilli to long slender rods and often are diphtheroid in appearance. Cells may be arranged singly, in parallel clusters or in chains. Colonies on blood agar or acid media, such as tomato juice agar, pH 5.0, are small (0.5 to 1.5 mm.) and may be smooth, rough or intermediate in texture.

The lactobacilli grow either aerobically or anaerobically and in either neutral or acidic media. These bacteria are highly fermentative, do not liquefy gelatin, reduce nitrates nor produce indole. A large number of species are recognized from the mouth

(oral lactobacilli), from the intestinal tract (particularly *Lactobacillus bifidus*) and from the human vagina (Döderlein's bacillus), milk and milk products and fermented foods. Classification is based in part upon the source of the cultures, in part upon fermentation and physiological reactions and upon immunological characteristics.

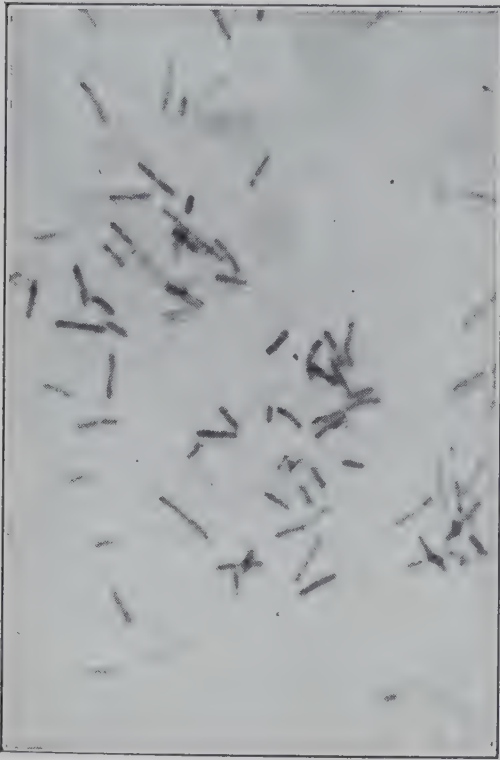


Fig. 168. Aciduric rods (Döderlein's bacillus) in vaginal smear. Epithelial cells are shown faintly in the background. (Magnification approximately  $\times 1,400$ .)



lactobacilli contain an immunologically active, type-specific carbohydrate. **Lactobacillus Acidophilus.** The aerobic parasitic lactobacilli are commonly designated as *L. acidophilus*. This organism has been cultivated from the intestine, the vagina (Döderlein's bacillus), and the oral cavity. These bacteria do not form a homogeneous group, so that subdivisions on the basis of colony morphology, fermentation reactions and immunological characteristics have been made. In the intestine, the number of lactobacilli is particularly great when the diet includes large quantities of milk or fermented milk foods. The flora of the normally acid vagina is comprised predominantly of lactobacilli. In the mouth, lactobacilli are particularly numerous in areas of dental decay and about the teeth. There is indirect evidence that the acidity produced during fermentation of these and other glycolytic bacteria is related to destruction of the tooth substance and hence dental caries or decay. The lactobacilli are increased in number when caries is active and they are found intimately associated with the lesions.

**Lactobacillus Bulgaricus.** *Lactobacillus bulgaricus* is closely related to *L. acidophilus*. This organism has been widely used in the production of bulgaricus cheese, which at one time was advocated as a health food to reduce intestinal fermentation. Acidophilus milk has similar properties and is frequently used as a health food, although its therapeutic value is highly questionable. The Boas-Oppler bacillus found in the stomach in partial obstruction and cancer of the stomach is a related bacterium.

**Lactobacillus (Bacteroides) Bifidus.** *Lactobacillus bifidus* is a highly pleomorphic, anaerobic bacterium which is found in the mouth and intestinal tract. It is characteristically present in the intestinal flora of nursing infants. This organism is closely related to the anaerobic nonsporulating *Bacteroides* as well as to the lactobacilli. It is closely related to *L. acidophilus*.

## LISTERELLA

The *Listerella* are small, nonsporulating, motile, gram-positive bacteria which are commonly classified into one species, *Listeria monocytogenes*. These organisms are of undoubted pathogenicity for man and animals, although infection is frequently recognized. They have been isolated most commonly from infections of the covering membranes of the central nervous system, i.e., meningitis, in man and animals, particularly sheep, and have been associated with abortion in cattle. The name *Listeria monocytogenes* is derived from the type of cellular response produced by infected rabbits. In these animals there is an increase in the large monocytes of the blood, which somewhat resembles mononucleosis in man. Indeed, *Listeria* has been isolated from human mononucleosis, although it is not accepted as the causative agent in the human disease.

*Listeria* is a small gram-positive rod which is motile by a terminal flagellum. The cells structurally resemble small diphtheroid bacteria. Colonies are small,

hemolytic on blood agar and are not unlike colonies of streptococci. Acid is produced from carbohydrates, but the bacteria are otherwise biochemically inactive. Growth occurs either aerobically or anaerobically at 37° C. The antigenic activity of these bacteria is incompletely known, although at least two types may be recognized by agglutination and precipitation tests.

### BARTONELLA

The *Bartonella* are small bacterial cells which are intracellular parasites of man and the lower animals. Although they are gram-negative, they stain with Giemsa's stain. They are cultivated with difficulty in tryptone-yeast medium. The best known members of the group are *Bartonella bacilliformis*, the causative agent of human bartonellosis (Oroya fever, verruga peruana, Carrion's disease), and *Bartonella muris*, a latent parasite of rats. The *Bartonella* resemble the *Rickettsia* in their small size and intracellular parasitism and recently have been classified in the order Rickettsiales.

Human bartonellosis may take either the acute form (Oroya fever), which is a febrile disease with a marked anemia, or the chronic cutaneous form (verruca peruana). *Bartonella* may be demonstrated within the red blood cells by Giemsa-stained smears or they may be cultured. There is enlargement of the spleen and lymph nodes during the acute stages and the reticulo-endothelial cells are actively phagocytic. In verruga peruana there is a pink macular eruption of the skin and nodules which are highly vascular and tend to hemorrhage and ulcerate. Oroya fever is fatal in from 20 to 40 per cent of cases, but verruga peruana is chronic and not fatal. Human bartonellosis is transmitted by several species of *Phlebotomus* flies (sand flies), and is sharply limited in its geographical distribution to that of these flies. The disease occurs only in tropical America in certain arid river valleys of the Andes region of Peru and Colombia. An attack of the disease appears to confer immunity against subsequent infection, but there is no specific therapeutic or preventive measure except personal protection against the flies.

### GLANDERS AND MELIOIDOSIS

The *Malleomyces* (*Pfeifferella*) are slender, gram-negative rods, which in some respects resemble the *Mycobacteria* and the *Pasteurella* or *Brucella*. The genus is comprised of two species, *Malleomyces mallei*, the glanders bacillus, described in 1882 by Löffler and Schutz, and *Malleomyces whitmori*, the causative agent of melioidosis, a glanders-like disease of man and rodents.

Glanders, which occasionally affects man, is an important disease of equine animals. In horses, infection results in formation of nodules and abscesses in the lymphatics, the lungs, the nose and other tissues (glanders) and in subcutaneous and cutaneous nodules (farcy). Glanders, which is the more acute form of the disease, is the form usually seen in mules and asses, and the fatality is high.



Chronic glanders and farcy an acute attack of the generalized disease is frequently observed terminally. The disease may be reproduced experimentally. Human infection, which is infrequent and is usually acquired from infected horses, is similar to that in horses and is highly fatal. Other domestic animals are relatively resistant to infection. The guinea pig is, however, susceptible to infection and is used in diagnostic tests. In this animal a generalized disease characterized by swelling and redness of the testes is produced (the Strauss reaction).

The glanders bacillus is a small nonmotile rod of irregular appearance which stains with difficulty. It is gram-negative and not acid-fast. Colonies are small, white and yellowish in color on agar medium or gray-green on blood agar. Growth is favored by glycerol or enriched media. Gelatin and glucose may be liquefied, but otherwise the glanders bacillus is biochemically inactive.

The mode of spread of glanders is uncertain, although evidence favors infection by the gastro-intestinal route in the systemic disease. Infection may occur through injuries of the skin in farcy and the nasal discharges and the feces of horses with glanders are infectious. Glanders has been widespread among horses throughout the world, but as a result of vigorous control measures it is a disease of decreasing importance. It is infrequent in the United States.

The diagnosis of glanders is made by means of (1) the observation of the local disease in guinea pigs inoculated with infectious material, and (2) the mallein test. Mallein is a concentrate of glycerol broth cultures of *M. mallei* homologous to tuberculin. In the subcutaneous test, fever and a local tender swelling develop in 24-48 hours in infected horses. In the conjunctival test, an intense inflammation of the eye follows installation of mallein into the conjunctival sac. The mallein test is highly specific and has great diagnostic significance. Precipitins and complement-fixing antibodies may also be demonstrated.

Neither an attack of glanders nor artificial immunization confers immunity against the infection, and in the infected animal the disease may become acute after a long period of chronic infection. Control has been accomplished by slaughtering infected animals.

Melioidosis, caused by *M. whitmori*, is a disease of rodents and man in the Orient and Pacific Islands. In man caseous abscesses are observed in many organs, the lung, liver, spleen, kidneys and lymph nodes. The epidemiology of the disease is not known with certainty, but presumably man is infected from rodents, possibly by contaminated food. The causative organism is motile, grows readily on ordinary media, liquefies gelatin and is fermentative. On glycerol broth, growth is rough and highly tenacious. Cultures inoculated into guinea pigs produce the Strauss reaction.

## PLEUROPNEUMONIA

The pleuropneumonia organisms are small, structurally bizarre forms which resemble the viruses in many respects but are like the bacteria in that they may

be cultivated on artificial media. Originally described as a virus responsible for bovine pleuropneumonia, organisms of the group have now been cultured from cattle, sheep, goats, dogs, rats, mice and man and have been isolated from sewage. They are known to be pathogenic in some instances, whereas members of the group appear to be commensals or saprophytes.

The pleuropneumonia organisms are extremely pleomorphic, particularly on solid media. The cells vary in size from minute virus-like particles to long filaments, globules and discs, all in the same culture. They stain with Gram stain but not with the usual bacteriological dyes. The parasitic pleuropneumonia organisms develop aerobically, usually less well anaerobically, and require serum or ascitic fluid enrichment. Colonies on solid media are microscopic in size (usually to 0.6 mm. in diameter) and usually require several days for development. Saprophytic members of the group are less exacting in growth requirements; otherwise they resemble the parasitic organisms.

Pleuropneumonia of cattle is a highly communicable disease which is important in many regions of the world. It is absent from North America. The disease is characterized by extensive pneumonia and pleurisy and occasionally by arthritis. The causative organism is restricted in its host range and, although naturally highly infectious, is experimentally reproduced with difficulty. Agalactia of sheep is a systemic disease caused by pleuropneumonia organisms. Infected animals of both sexes develop arthritis and often abscesses of the joints and a skin eruption. As the name implies, mastitis occurs in lactating females. The pleuropneumonia organisms of rats have been subject to extensive investigation. Three different types, known as  $L_1$ ,  $L_3$  and  $L_4$ , have been isolated from normal and chronically infected lungs ( $L_3$ ), from spontaneous arthritis, and in intimate association with bacterial parasites ( $L_1$ ). The  $L_1$  organism is nonpathogenic and is usually isolated with *Streptobacillus moniliformis*. In the case of the pleuropneumonia organisms have been interpreted by some investigators as being a stage in the life cycle of this organism and other bacteria. Isolation in pure culture is, however, against this point of view. In mice, pleuropneumonia bacteria have been found in the central nervous system, particularly in "rolling discs" and in the lungs. Experimentally, pure cultures produce severe arthritis in mice. Although pleuropneumonia organisms have been isolated from human sources, their relationship to disease has not been established. The saprophytic members of the group isolated from sewage and decomposing matter have not been shown to be pathogenic.



## THE SPIROCHETES

The spirochetes are a group of spiral microorganisms which possess properties relating them to both the bacteria and the protozoa. They are classified separately from the true bacteria in the order Spirochaetales. Ehrenberg (1838) described saprophytic spirochetes from water. Later, as other similar organisms were recognized, the group was enlarged to include those spiral micro-

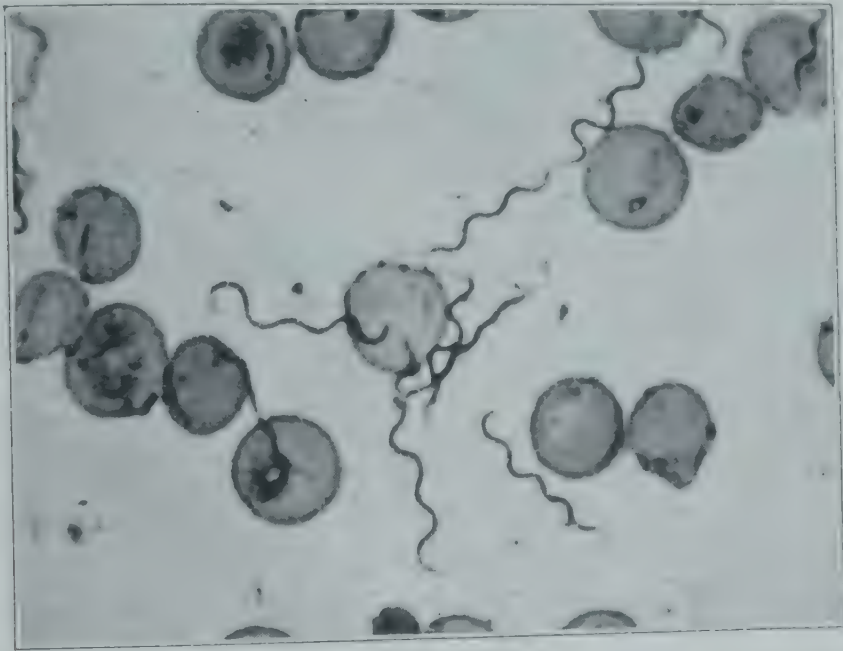


Fig. 169. *Spirochaeta duttonii* in the blood. Photomicrograph of blood smear. (Magnification approximately  $\times 1,200$ .) (Kral.)

organisms which are motile by means of a screw-like motion of the cell rather than by means of flagella. The pathogenic and parasitic members of the group include *Treponema pallidum* of syphilis (Schaudinn and Hoffmann, 1905), *Treponema pertenue* of yaws (1905), *Leptospira icterohæmorrhagiae* of Weil's disease (1915), *Borrelia recurrentis* of relapsing fever (Obermeier, 1873) and *Borrelia vincentii* of fusospirochetal disease (Vincent, 1906).

**Morphology and Classification.** The morphology of the Spirochaetales has been described in Chapter 6. Like all members of this order, the pathogenic species have a spiral form which varies from small rigid coils to large irregular

spirals. In preparations of the living cells motility is observed in all in which, unlike that of the bacteria, is related to screw-like rotational or movements of the cell body. A few of the parasitic spirochetes have been cultivated on highly complex artificial media, such as Noguchi's *Leptospira* 1 whereas others are cultivated with difficulty or only in the presence of tissue (tissue culture or embryonated eggs). The internal structure of spirochetes is similar to that of the bacteria, and cell division is by simple transverse division. Their physiology is relatively unknown. Special staining methods are commonly used for demonstrating spirochetes. Smears stained by Giemsa's method are satisfactory for many species, such as *Bor. recurrentis*, which may be found in blood smears from patients with relapsing fever. In other instances, the silver impregnation method, in which the organisms become impregnated with silver and appear black, is most successful and is employed for the demonstration of *T. pallidum* in syphilitic lesions. The observation of the living motile spirochetes in fresh preparations of infected tissues by means of the darkfield microscope is also extremely useful.

TABLE 15. CHARACTERISTICS OF THE PATHOGENIC SPIROCHETES

GENUS	CHARACTERISTICS	SPECIES	DISEASE
<i>Treponema</i>	4 to 16 $\mu$ in length by 0.25–0.3 $\mu$ in diameter; ends tapered; coils regular, rigid and 1 $\mu$ in depth; axial filament doubtful; stain with difficulty; disintegrated by bile salts and saponin; movements rotational and bending	<i>T. pallidum</i>	Syphilis
		<i>T. pertenue</i>	Yaws (frank)
		<i>T. carateum</i> ( <i>T. herrejoni</i> )	Mal de pin (carate, pin)
		<i>T. macrodentium</i>	Normal parasite of mouth
		<i>T. microdentium</i>	
		<i>T. minutum</i>	Normal gerbil parasite
<i>Leptospira</i>	4 to 16 $\mu$ in length by 0.3 $\mu$ in diameter; ends tapered and one or both curved or hooked; coils 0.5 $\mu$ in depth, small; disintegrated by bile salts but resistant to saponin; movements rotational and lashing. Cultivation: 10 per cent serum in Ringer's solution or in 2 per cent agar	<i>Lept. ictero-haemorrhagiae</i> <i>Lept. canicola</i>	Infectious disease (Weill's disease)
		<i>Lept. hebdomadis</i>	Japanese spring fever
<i>Borrelia</i>	8 to 16 $\mu$ in length by 0.3 to 0.5 $\mu$ in diameter; pointed ends with terminal filament; loose, irregular coils; stain readily; movements slowly rotational and lashing; disintegrated by bile salts, less readily by saponin	<i>Bor. recurrentis</i>	Relapsing fever
		<i>Bor. vincentii</i>	Fusospirochetosis (Vincent's disease)
		<i>Bor. theileri</i>	Equine relapsing fever
		<i>Bor. gallinarum</i>	Septicemia of chickens
		<i>Bor. anserina</i>	Febrile disease of geese
	2–5 $\mu$ in length; flagellate	<i>Bor. muris</i> ( <i>Spirillum minus</i> )	Rat-bite fever



The spirochetes are generally somewhat less resistant to deleterious agents than are the bacteria. They are readily destroyed by heat, chemical disinfectants and drying. *Treponema pallidum* survives for only a few hours outside the animal body, whereas other spirochetes may, if moist, survive for considerable periods of time. Spirochetes parasitic and pathogenic for man and the higher animals are included in the genera *Treponema*, *Leptospira* and *Borrelia*. The classification is not entirely satisfactory since *Borrelia* are very similar to *Treponema* morphologically and physiologically. Species are separated largely on the basis of habitat, disease production and geographical distribution, rather than on physiological and immunological characteristics, as are the bacteria.

## TREPONEMA

**Syphilis.** Syphilis is the most important and widespread spirochetal disease of man. The origin of the disease is a highly debatable point in medical history. Some authors believe it had been present in Europe long before it was recognized as an epidemic form in 1494, but there is some evidence to suggest that syphilis was introduced from the New World by sailors returning from Columbus' explorations. In any case, a highly virulent infection, variously known as the Italian disease, the French disease and *Morbus gallicus*, became widespread during the sixteenth century. The modern name is derived from a poem, *Syphilis sive Morbus Gallicus*, written by Fracastorius in 1530 in which the symptoms of acute syphilis are described. The venereal or sexual transmission of syphilis was not early recognized, and the causative spirochete was not demonstrated in the genital lesions until 1905 (Schaudinn and Hoffmann). Therefore *T. pallidum* has been recognized in the body tissues in early and late syphilis and in experimental syphilis. Chemotherapy of syphilis was introduced with the demonstration by Ehrlich of the spirocheticidal action of salvarsan.

Infection with *T. pallidum* is in nature a human disease; it may be transmitted experimentally to a few other primates and to rabbits. The human disease is generally acquired by personal contact with an infected person during the active primary and secondary stages of the disease (early syphilis). The contact is usually, but not necessarily, sexual. Transmission may take place congenitally from infected mother to child during pregnancy, and indirectly through the use of contaminated towels, instruments and other articles (see chapter 43). Transmission is generally direct, since *T. pallidum* does not long survive outside the body.

Syphilis is a generalized disease which may affect almost any tissue in the body. The skin and mucous membranes, the cardiovascular, gastro-intestinal and genito-urinary tracts, the bones, the central nervous system, the lymph nodes, liver and spleen may contain lesions of the early or late disease. Consequently there are many manifestations of syphilis. The manifestations of congenital syphilis are in many respects different from those of acquired infection.

The lesions of acquired syphilis are best discussed as those characteristic of the early disease (primary and secondary stages) and those found in the late infections (tertiary stage). The initial or primary lesion is the Hunterian hard chancre, a firm, indurated, painless, usually single ulcer or nodule which develops within a few weeks at the site of infection and is accompanied by enlargement of the regional lymph nodes. *Treponema pallidum* is readily demonstrated in the fluid of the chancre, but, as shown by the progression of the disease, is not limited to this skin lesion. Within four to six weeks the chancre heals spontaneously and is followed immediately or after a second incubation period of variable duration by the secondary lesions. At this time there are general symptoms of infection, such as fever, malaise, weakness and pain, and a generalized eruption of the skin and mucous membranes.

The spirochetes are generally distributed throughout the body during secondary syphilis and are abundant in the cutaneous lesions as well as in the deeper tissues. The lesions of the skin and mucous membranes are thus infectious. Other symptoms may be related to infection in specific locations within the body. The late or tertiary and visceral stages of syphilis begin approximately 1 to 2 years after infection, but these may not be apparent for many years. The tertiary disease is characterized by the presence of small numbers of *T. pallidum* within the tissues and by formation of chronic ulcers and inflammatory lesions known as gummata, which are destructive of the normal body tissues. The chronic inflammatory lesions of late syphilis are of the granulomatous type similar in structure to the lesions of tuberculosis. Gummata may be present in any tissue in the body, as may also other destructive lesions. Thus syphilis of the cardiovascular system and of the brain in paresis or general paralysis and the spinal cord in tabes dorsalis are highly important manifestations of late syphilis, in which there is destruction of the tissue by direct spirochetal infection. Secondary formation is prominent in late syphilis. Although the characteristics of syphilis are different in the different stages, there may be overlapping, or the stages may pass insensibly from one to the next.

Congenital syphilis is infection acquired by the child from the infected mother during pregnancy. Transmission takes place through the placenta and may occur at any time during the mother's infection, but it most likely occurs during relatively early maternal infections. The syphilitic fetus may be stillborn, or the newborn child may be obviously infected at birth. In other instances, particularly in infections acquired during the late disease in the mother, the onset of symptoms in the child may be delayed for months or years. There is no primary stage in congenital syphilis, and the lesions, while often acute and septicemic, more closely resemble the gummata of late acquired syphilis. Malformations and destructive lesions are frequent.

**Immunity.** Resistance to a second infection with *T. pallidum* appears to be an infection immunity which may be incomplete; that is, in the presence of syphilis a second infection does not result in production of a second chancre or exacerbation of disease, although spirochetes may enter the tissues. There is no



ence that immunity effects a cure in untreated syphilis; the spirochetes remain latent indefinitely with recurrence of symptoms and slowly progressive destruction. Following treatment, however, cure may be effected, and infection may then take place with the development of typical syphilis. There is evidence of natural individual or racial immunity to syphilis. The disease is, however, less virulent at the present time than during the early years following its recognition.

Study of antibodies against the spirochetes in syphilis is exceedingly difficult. Our knowledge is very incomplete, largely because of the delicacy of the spirochete and the uncertainty of its cultivation or of its infection in animals. Immunological studies, however, revealed that in syphilitic infection an antibody (reagin) is present in the blood serum, and that this antibody together with infected-tissue extracts (antigen) fix complement and form a precipitate. It thus became possible to detect syphilis by serodiagnostic or blood tests using complement-fixation and precipitin reactions (see page 268). It is, however, necessary to use infected tissues for preparation of antigen since syphilitic serum will fix complement and form a precipitate with suitable extracts of normal tissues. The nature of the syphilitic reagin is poorly understood. The serodiagnostic tests are of great value in the diagnosis of the disease and, with the exception of a persistently positive reaction in yaws, are positive in few other conditions. On the other hand, the reaction is certainly not a specific one between *Treponema* and antibodies against it. Different theories have related syphilitic reagin to antibody against the body tissues (isoantibody) to antibody against antigens of the nonspecific heterophile type, which are found in many animals and in some bacteria, or to a "pathological protein."

Reagin is rarely present until at least two weeks after the onset of symptoms, although during the latter part of the primary stage serological tests are positive in about 40 per cent of patients. Diagnosis of primary syphilis is, therefore, better made by the demonstration of *T. pallidum* in fluid expressed from the chancre by means of the darkfield microscope than by serological tests. On the other hand, the serological tests are the most reliable single method for diagnosis in later stages of the disease. It is agreed that these tests are accurate in approximately 85 to 90 per cent of individuals during the secondary stage and are somewhat less reliable in late infections, although in the opinion of some investigators the accuracy is considerably greater. The complement-fixation reaction on the spinal fluid is of primary importance in the recognition of syphilis of the central nervous system and is positive in approximately 100 per cent of cases. Serological tests usually become negative during treatment of syphilis, although a small percentage of patients continue to have reagin in their blood following adequate treatment and the disappearance of other evidence of infection. Infants of mothers having a positive blood test for syphilis are also found to give a positive reaction which soon disappears unless the child is congenitally infected.

Several tests are employed for the serological diagnosis of syphilis, the best

known of which are the Kolmer complement-fixation (Wassermann) test, the Kahn, the Kline, the Hinton and the Eagle precipitation tests. In the hands of experienced serologists these tests give a high degree of correlation; they are, however, carefully balanced reactions, so that technically inaccurate tests do not give falsely positive reactions for syphilis. Occasionally false positive reactions are obtained for other reasons, in which case the Kahn verification test performed at  $37^{\circ}$  and  $1^{\circ}$  C is of some value. Syphilitic reagin gives a strongly positive precipitation reaction at  $37^{\circ}$  C, whereas the nonsyphilitic reactions generally are strongly positive at  $1^{\circ}$  C. Although the antigens used in these several tests differ in some respects, all contain the alcohol-soluble and acetone-insoluble fraction of beef heart muscle and cholesterol. In the Kolmer-Wassermann reaction the suitably diluted antigen and the patient's serum are incubated with complement (guinea-pig serum), after which the hemolytic system of sheep red blood cells and antihemolysin is added, and the mixture is incubated a second time. As in all complement-fixation reactions a positive test is indicated by the **absence** of hemolysis (see page 270). In the precipitation tests a flocculation occurs when syphilitic serum is mixed with antigen diluted in saline.

**Treatment.** The treatment of syphilis is entirely a matter of chemotherapy. Until recently, treatment required long series of injections of organic arsenic compounds, particularly those of the arsphenamine (salvarsan or 606) series, and compounds of certain heavy metals, particularly bismuth, and in some instances malaria or artificial fever therapy. Although the infectivity of early syphilis is rapidly eliminated by this method, prolonged treatment is necessary for the control and cure of the disease. With the introduction of penicillin a remarkably safe and effective method of treatment has been developed which promises to replace entirely the older methods. It should be pointed out that, as in the other communicable diseases, prevention of syphilis is in the long view preferable to treatment. The epidemiological and community aspects are discussed in Chapter 43.

**Yaws (Frambesia).** Yaws is a nonvenereal spirochetal disease of man caused by *Treponema pertenue*, which is limited in geographical distribution to tropical zones. The disease is prevalent in tropical America, Africa and parts of Asia, the East Indies, West Indies and Pacific Islands, but is uncommon in India and China. Yaws is more common in males and is predominantly a disease of children. Infection occurs primarily through cuts, abrasions or other injuries of the skin, or by contact with freshly contaminated articles or open cutaneous lesions, although house flies of the genus *Hippelates* may transmit the *Treponema*. These flies feed on the skin lesions and may harbor the causative agent for several hours.

Like syphilis, yaws is characterized by the formation of destructive granulomatous lesions. The lesions are particularly numerous on the skin and tend to ulcerate, often with severe deformity and scarring. The disease begins by the development of a single cutaneous lesion known as a "mother yaw." This is followed after some weeks by secondary and tertiary stages of generalized infection, which are very similar to those of syphilis. Indeed, the two diseases are closely related, and



er differentiation may be exceptionally difficult. The serological tests for syphilis are uniformly positive in yaws and the spirochetes are indistinguishable. Experimental infections are also similar. The laboratory diagnosis of yaws is made by demonstrating spirochetes in the lesions and by serological tests. The treatment is similar to that of syphilis.

Bejel is an infection with *Treponema* which is not clearly distinguished from yaws and syphilis. The disease is nonvenereal and has been reported among the desert-living Arabs, predominantly among children.

**Pinta.** Pinta affects the dark-skinned races of tropical America, particularly Mexico and Colombia, and is characterized by permanent loss of pigmentation in patchy areas of the skin. Pinta begins with the development of a single, later generalized, cutaneous eruption followed by the appearance of areas of vari-colored skin lesions which eventually become depigmented. The cause of pinta was unknown until 1938 when treponemata were demonstrated in preparations from the skin lesions. *Treponema carateum* (*T. herrejoni*) is now established as the causative agent, although the means of transmission remains obscure. Serological tests are usually positive in the late stages of the disease and may be helpful in its recognition. Treatment is identical with that of syphilis.

## BORRELIA

Spirochetes of this genus closely resemble the causative agents of syphilis, yaws and are sometimes classified as *Treponema*. The results of infection with these organisms are, however, very different from those caused by recognized treponemata. The *Borrelia* tend to give rise to acute febrile illnesses, chief among which are the relapsing fevers. The first etiological agent of this group to be described was *Borrelia recurrentis*, isolated by Obermeier (1873) in European relapsing fever. Since that time spirochetal relapsing fevers have been described from many parts of the world, such as those due to *Bor. duttonii* and *B. kochii* in Africa, American relapsing fever (*Bor. novyi* and *Bor. turicata*) and similar diseases in India (*Bor. carteri*) and South America (*Bor. venezuelensis*). In the United States, foci of relapsing fever are recognized in several northern and southern states. The spirochetes of relapsing fever are heterogeneous serologically, and in a single infection the spirochetes isolated from different lesions have been found to be antigenically different. *Borrelia vincentii* is found in the oral cavity (Plaut-Vincent angina) and elsewhere in fusospirochetal disease. *Borrelia muris* (*Spirillum minus*) of rat-bite fever is more closely related morphologically to the spiral bacteria, since it possesses flagella and is smaller than the other spirochetes. However, rat-bite fever due to this organism is similar to the others of spirochetal origin.

**Relapsing Fever.** The relapsing fevers encountered in different geographical areas are clinically very similar and are characterized by paroxysms of fever with intervening periods without symptoms. The disease usually begins suddenly with the onset of high fever and chills which last several days, followed by a symptom-

free period of one to three weeks and then a return of symptoms. The disease is self-limiting, usually disappearing after two and occasionally more bouts of fever. A skin rash may be present in the initial attack but is usually absent thereafter. As noted above, the relapses appear to be related to the production within the body of immunologically different spirochetes, which are able temporarily to overcome the immunity developed during the initial attack. The diagnosis is made by demonstrating spirochetes in the blood, either in stained (Giemsa) smears or by the darkfield microscope. It is doubtful that the relapsing fevers encountered in different geographical regions are different diseases, or that the spirochetes are truly different species. There are, however, differences in the epidemiology of these diseases.

The relapsing fevers are all transmitted by blood-sucking insects or ticks. European relapsing fever is transferred from man to man by the human body louse, and less conclusive evidence incriminates the bed bug. The disease occurs, therefore, under conditions such as crowding, lack of cleanliness and indoor living, which favor transference of the louse from person to person. The spirochetes are present within the tissues and fluids of the louse and are introduced into the human body through bites or injuries of the skin contaminated by crushed infected lice. They are not transmitted by the louse during biting. Elsewhere relapsing fever is primarily a tick-borne disease. Ticks of several species, all members of the genus *Ornithodoros*, have been found capable of transmitting infection. Relapsing fever in Africa is transmitted by *O. moubata*, in North America by *O. hermsi* and *O. turicata* and in Central America by *O. talaje*. These ticks live in the walls and the floor of human habitations, in decaying wood, caves and about animal burrows. It is of great interest that, once infected, the female tick may transmit the spirochetes to successive generations, which in turn are infectious. The spirochetes are found in secretions of the coxal gland of the tick and contaminate the bite during feeding. The saliva and feces of the tick may also be infectious.

The clinical course of relapsing fever may be interrupted and the infection cured by the antisyphilitic arsenical drugs, and recent reports indicate the successful use of penicillin. Control of the disease depends upon reducing or eliminating infestation with the body louse in the case of the European type and in the other diseases in control or avoidance of the tick vectors. Human habitations may be fumigated to eradicate ticks, but otherwise prevention is largely a matter of personal protection in infected areas.

**Vincent's Angina and Fusospirochetal Disease.** Vincent's angina of the mouth and fusospirochetal disease of other tissues are foul-smelling, necrotic infections associated with the presence of abundant fusiform bacilli and the spirochete *Bor. vincentii*. The diagnosis of fusospirochetal disease is made by the demonstration of these organisms in smears of material from the lesions stained by Gram's or Giemsa's methods. It is not clear whether Vincent's angina or stomatitis is a true infectious disease caused by these organisms or whether the bacteria are secondary invaders and the underlying cause a nutritional de-



ency or some other factor. It is interesting in this connection that, although hemics of Vincent's angina are a matter of record, normal human beings are resistant to infection. There is little doubt, however, that fusospirochetal infection may be present in lung abscess and at times in gangrenous mixed infections of other tissues.

**Spirochetal Rat-Bite Fever.** A relapsing type of fever caused by *Borrelia muris* (*Spirillum minus*) may follow the bite of rats, dogs, cats, squirrels and certain other animals, or may be acquired by handling infected animals. The disease begins several days to two weeks following the bite and is characterized by repeated bouts of fever at regular intervals, during which the causative organism may be found in the local lesion, the lymph nodes and less regularly in the blood. Spirochetal rat-bite fever is world-wide in distribution; it is infrequently reported in this country. The bacteriological diagnosis is best made by the inoculation of previously uninfected mice or guinea pigs, although the demonstration of spiral organisms in the blood or tissue fluids by stained smears and darkfield microscopic examination is frequently of value. *Borrelia muris* is a natural parasite of animals. The antispiochetal arsenical compounds are effective in the treatment of rat-bite fever.

## LEPTOSPIRA

Leptospira pathogenic for man and animals are world-wide in distribution. and similar, presumably saprophytic, organisms have been described from several sources. The human diseases caused by leptospira are clinically very similar but differ in severity, the epidemiological details and in the causative organisms. The most commonly recognized and severe human infection is infectious jaundice (Weil's disease), which is usually caused by *Lept. icterohaemorrhagiae*, but which may result from infection with *Lept. canicola*. In Europe, swamp fever occurs in swampy areas in agricultural workers who become infected with *Lept. pygmaea*, and in Japan seven-day fever is a mild autumnal fever resulting from infection with *Lept. hebdomadis*. Among animals leptospiral infection occurs naturally among rats, mice, dogs, cats and certain other domestic and wild animals. Infection of rats, mice and dogs appear to be particularly important with regard to the occurrence of human leptospirosis.

Saprophytic *Leptospira*, usually referred to as *Lept. biflexa*, are widely distributed in the water of ponds, pipes, mines, etc. *Leptospira biflexa* is non-pathogenic and, although morphologically identical, is antigenically different from *Lept. icterohaemorrhagiae*.

**Spirochetal Infectious Jaundice (Weil's Disease).** Human leptospirosis is typically an acute febrile disease which begins suddenly after an incubation period of one to two weeks. In the more severe and typical infections (Weil's disease) jaundice and interference with function of the liver are prominent by the end of the first week, at a time when the fever is returning to normal. Relapses may occur. Fatality of the disease is generally low but may be as high as 25 to 30 per cent of cases.

During the acute stages of Weil's disease examination of the patient's blood sometimes reveals *Leptospira*. However, the *Leptospira* are commonly absent from the blood after the first week and are found in the tissues, particularly the kidney. The urine is infectious. Direct examination of blood for spirochetes is unsatisfactory so that the bacteriological diagnosis is made by the culture of the organisms, inoculation of guinea pigs or hamsters, and by serological tests. In infected animals the organisms are present in blood. Both spirocheticidal antibodies and agglutinins are present in convalescent serum, and the agglutination test is of particular diagnostic value. The agglutinin titer may be exceptionally high, 1:10,000 to 1:30,000, although values over 1:100 are significant.

Infection in man is secondary to the presence of *Leptospira* in the local animal hosts. Wild rats are the chief reservoirs of infection with *Lept. icterohaemorrhagiae* and *L. canicola*. Surveys indicate that generally 50 to 90 per cent of wild rats harbor *Leptospira* and as many as 25 per cent of dogs are infected. The organisms are present in the rat kidney and are eliminated in large numbers in the urine. Demonstration of infection may be made by direct darkfield examination of urine or kidney emulsion or by animal inoculation tests. The infection is transmitted to man in water contaminated by rat urine; invasion may occur either through injuries and abrasions of the wet skin or by ingestion. Weil's disease has occurred in epidemics among bathers and is frequent among workmen subject to exposure to contaminated water. Thus leptospirosis is an occupational hazard of miners, sewer workers and fish workers. During World War I the disease was present among soldiers occupying wet, rat-infested trenches on the Western Front.

Swamp fever of Europe and seven-day fever of Japan are relatively less severe infections, and jaundice is a less prominent feature. Epidemiologically the diseases differ in the animal reservoirs and hence in the human population groups affected. Several species of wild mice harbor *Lept. grippotyphosa* and cause swamp fever. *Leptospira hebdomidis* of seven-day fever is found in the field mouse, as are several other agents of similar diseases in the Orient. Swamp fever and seven-day fever occur particularly among agricultural workers.

The treatment and prevention of leptospirosis involves rat eradication, protection of beaches, avoidance of infested areas and personal protective measures. Immune serum and vaccination appear to be of some value. However, arsenic drugs are ineffective, and the value of penicillin is not clearly established.



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### THE RICKETTSIAL DISEASES

The rickettsial diseases are a group of acute, often highly fatal diseases caused by small, coccobacillary microorganisms, the *Rickettsiae*, which were first described by Ricketts (1909) in Rocky Mountain spotted fever. With the notable exception of epidemic typhus fever, the rickettsial diseases are maintained in nature in their arthropod vectors and vertebrate animal reservoirs, so that man is secondarily infected. The rickettsial diseases are customarily divided into the typhus fever group, the spotted fever group, the tsutsugamushi group, the Q fever group and several unclassified diseases which will be considered separately. The causative rickettsiae of these several groups of diseases do not differ in morphology, resistance or cultivability. Their physiology is almost completely unknown. Each may be recognized, however, by pathogenicity for man and animals, and the pathology, epidemiology and immunology of the diseases which they cause.

**Cultivation and Vaccination.** Since the rickettsiae may not be cultivated in cell-free, artificial media as may the bacteria, methods for obtaining large numbers of these microorganisms assume importance in the development of vaccines and in maintaining the agents in the laboratory. Several methods of cultivation are available which are in some respects different for the different rickettsiae. The first method was that of Spencer and Parker, who obtained rickettsiae of Rocky Mountain spotted fever from the viscera of infected ticks in sufficient numbers to prepare a vaccine. The rickettsiae in the ground tick tissue were killed with phenol. In epidemic typhus fever a similar but more difficult method was employed for the production of vaccine from infected louse excreta. Later, the rickettsiae of murine typhus were obtained in large quantities by infecting rats, whose resistance had been reduced by x-irradiation. Formalinized suspensions of rickettsiae thus obtained have been widely used as vaccines for the prevention of murine typhus. The third method is that of tissue cultures. The organisms of typhus and spotted fever have been cultivated in this way. However, the most successful and practical method of obtaining rickettsiae for vaccines and for study is that of growing them in the yolk sac of the developing (10-day) chick embryo. The rickettsiae multiply in the cells of the embryo, particularly those of the yolk sac, and cause death of the chick after 3 to 5 days. Vaccine is prepared by treating the rickettsiae from the ground-up embryonic tissues suspended in saline with phenol and formalin. Chick embryo vaccine is

now widely used for protection against typhus fever and is successful against spotted fever, but less so in tsutsugamushi disease.

The vaccines most widely used at present are typhus chick embryo vaccine and the Spencer-Parker spotted fever vaccine, which are usually given in a series of weekly injections, with booster injections at intervals of several months. Administration is limited to persons exposed to special risk and is not recommended for the general population.

**Classification.** The general relationships of the rickettsiae to the bacteria and the viruses have been discussed in Chapter 8. Intracellular parasites which

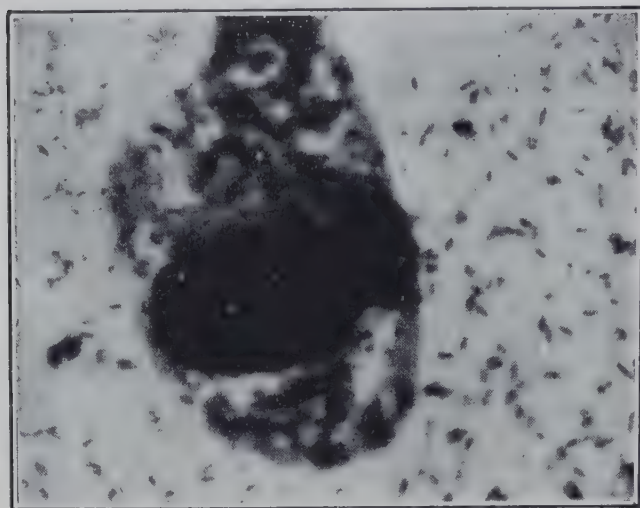


Fig. 170. *Rickettsia (R. prowazeki)* of typhus fever ( $\times 1,500$ ). (From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Appleton-Century-Crofts, Inc.)

are morphologically typical rickettsiae are widespread among arthropods and are for the most part nonpathogenic and incompletely described. These organisms are unclassified. The pathogenic rickettsiae are also parasites of arthropods and, excepting the body louse, are nonpathogenic for these animals. Indeed, there is in their lack of pathogenicity and in their hereditary transmission in ticks considerable evidence for the view that ticks and the related mites are the original hosts of the rickettsial pathogens.

The rickettsiae pathogenic for man are classified into species on the basis of pathogenicity for man and animals, epidemiology and arthropod vectors and immunological reactions, such as cross-protection in infected animals, complement-fixation tests and the Weil-Felix agglutination test. In general, the rickettsiae of the spotted fever group of diseases are tick-borne, produce similar pathology in infected guinea pigs and are immunologically related. With the exception of *Rickettsia conori* of boutonneuse fever, these organisms are all classified as *Rickettsia rickettsii* (formerly *Dermacentroxenus rickettsii*). In the typhus group, the classical epidemic or European typhus differs from endemic or murine typhus epidemiologically. The causative organisms are commonly classified as a single species, *Rickettsiae prowazeki*, although varieties *R. prowazeki prowazeki* and *R. prowazeki mooseri* of classical and murine diseases, respectively may be recognized on the basis of epidemiology and minor differences in animal pathogenicity and immunology. The diseases in man are identical. The mite-borne diseases of the tsutsugamushi group also are caused by immunologically identical agents and differ only in severity and in animal reservoirs in the different geographical areas. These rickettsiae are generally known as *Rickettsia orientalis* (*R. tsutsugamushi*, *R. nipponica*). The rickettsiae of Q fever



ferent geographical areas are immunologically closely related and are classified as *Rickettsia* (*Coxiella*) *burneti* (*R. diaporica*). The agent of trench fever has not been completely studied but is named *Rickettsia pediculi* and the name *Rickettsia akari* is proposed for the newly recognized agent of rickettsialpox. **Pathogenicity.** With the exception of the louse, which succumbs to typhus infection, no disease is produced in arthropods by rickettsia. In ticks they are transferred from one to another generation from the infected female without causing any ill effects in the progeny. In vertebrates, rickettsiae naturally infect many species of animals. *Rickettsia ruminantium* is strictly an animal pathogen, giving rise to heartwater disease of cattle, sheep and goats in Africa. The agent is similar to other rickettsiae in its parasitization of blood vessels and pathology and is transmitted by ticks of the genus *Amblyomma*.

The rickettsiae of the spotted fevers are infectious for many rodents, rabbits, cats, dogs, the opossum and monkeys. The spotted fever group may be distinguished from other rickettsiae by the severe scrotal infection and swelling produced in guinea pigs following inoculation by any route. A number of species of ticks of the genera *Dermacentor*, *Haemaphysalis*, *Amblyomma* and *Rhipicephalus* are either known or potential vectors of the spotted fevers. In classical murine fever, *R. prowazeki* is transmitted from man to man by the human louse (*Pediculus humanus*). There are no lower vertebrate hosts. Murine typhus, on the other hand, is endemic in rodent populations, is transmitted by rodent fleas, lice and mites and only sporadically affects man. The rickettsiae of typhus fever are infectious for man, many rodents, monkeys, the dog, and the opossum. Strains of murine rickettsiae differ from classical strains in being more pathogenic for laboratory animals; and in the guinea pig they produce a mild scrotal reaction after intraperitoneal inoculation. The human body louse develops a fatal infection with *R. prowazeki* and dies from infection of the intestinal wall. The sugamushi group of rickettsiae typically give rise to inapparent infections in guinea pigs, but some strains appear to be pathogenic for monkeys and nutritionally deficient guinea pigs. Scrotal swelling does not occur. *Rickettsia burneti* of human origin never produces a febrile reaction in experimentally infected guinea pigs and a mild pneumonia in monkeys and mice, but it produces inapparent infections in other laboratory animals. Naturally infected ticks have been recovered, and the agent is transferred through the eggs of infected females. *Rickettsia akari*, the agent of rickettsialpox, has been recovered from the naturally infected house louse and from the rodent mite, *Allodermanyssus sanguineus*. Experimental infection resulted in illness in mice and guinea pigs with the production of a mild scrotal reaction in the latter animals.

The rickettsiae are typically intracellular parasites, although they are at times found in the body fluids and excretions. Multiplication occurs within the body cells, particularly the cells of the small blood vessel walls, such as the endothelium. Injury and infection of these vessels results in thrombosis of the vessel lumen, interruption of blood supply and death of the tissue, inflammation and hemorrhage. Infection of the skin accounts for the maculopapular or hemorrhagic

rhagic rash of many human infections, and lesions in the brain are particularly serious. The scrotal reaction in guinea pigs results from localization of rickettsiae in this area with tissue damage.

**Immunity.** Recovery from one rickettsial infection is followed by a lasting immunity against reinfection with the same agent, and is associated with the presence of specific antibodies in the blood. These antibodies may be demonstrated by animal protection tests, phagocytic tests, agglutination and complement-fixation tests. Immunity is specific in that animals convalescent from infection with one agent are resistant to subsequent infection with the same or closely related rickettsiae, but not to unrelated species. The agglutination and complement-fixation reactions, however, may reveal antigenic differences between closely related strains which are not apparent in the animal protection tests. The rickettsiae of the typhus and spotted fever groups are unrelated by the animal protection test, and some further differences between classical and murine typhus strains are revealed by slight differences in protection and by complement-fixation reactions.

Since the observation by Weil and Felix that the blood of typhus patients agglutinates certain strains of *Proteus vulgaris*, the **Weil-Felix reaction** has become of great value in the diagnosis of rickettsial diseases. The reaction, which is an agglutination test using the patient's serum and the *Proteus* culture, is strongly positive in the typhus group and tsutsugamushi group of rickettsial infections but is less positive in the spotted fever group and negative in the Q fevers and rickettsialpox. The reaction appears to depend upon the presence of common antigen in the rickettsiae and *Proteus* organism and is not indicative of *Proteus* infection in the rickettsial fevers. The bacterial antigen is an O or cellular antigen and the strains of *Proteus* are known as OX strains. The Weil-Felix reaction has differential value in that antigenically different strains of *Proteus* are agglutinated by serums from different rickettsial infections. Thus serums from typhus patients strongly agglutinate *Proteus* OX-19, give some reaction with OX-2 strains and are negative with OX-K strains; serums from tsutsugamushi fevers are negative with OX-19 and OX-2 strains, but strongly agglutinate *Proteus* OX-K. Spotted fever serums give some reaction with all three cultures, but the titers are uniformly low.

### THE SPOTTED FEVER GROUP

The spotted fevers are a group of immunologically closely related tick-borne rickettsial infections. Some of the diseases of this group, however, do have certain clinical and epidemiological differences from classical Rocky Mountain spotted fever and the rickettsiae of fièvre boutonneuse and Kenya fever (*R. conchata*) have antigenic differences as demonstrated by the complement-fixation reactions.

Diseases of this group vary in severity from mild to highly fatal infections. Thus the mortality of spotted fever in the United States varies from less than 5 per cent to as high as 90 per cent in different areas. It is often stated that spotted fever in the eastern United States is milder than the western disease. However,



ographical variations are not constant and highly virulent strains of rickettsiae can be isolated in both regions. The mortality is generally slightly less than 20 per cent. The fatality of São Paulo fever is also high, but that of fièvre boutonneuse is low.

The incubation period of spotted fever is between 2 and 14 days and the disease usually lasts two to three weeks. There is high fever, severe malaise and prostration, and the appearance of a pink or rosy skin rash which later becomes hemorrhagic on all parts of the body, including the face, hands and feet. There are symptoms of gastro-intestinal, respiratory and central nervous system involvement. Fièvre boutonneuse and South African tick fever clinically resemble spotted fever, except that there is an initial lesion (the tache noire), which appears at the site of the tick bite before development of the generalized rash. There is no initial lesion in spotted fever, São Paulo typhus or Kenya fever.

Rocky Mountain spotted fever, which was first described from the Rocky Mountain region, is widespread in the rural United States, but it is particularly prevalent in mountainous regions of the West and in the South Atlantic States.

São Paulo typhus and the spotted fevers of Brazil and Colombia extend the geographical area of this group into South America. Fièvre boutonneuse is present in the countries bordering the Mediterranean and Black seas, where sporadic epidemics are reported, and Kenya fever occurs in Africa.

Rickettsiae of the spotted fevers are hereditarily transmitted from one to the next generation of ticks, without injury to the tick. These arthropods, which feed on mammals in the larval, nymphal and adult stages, are capable of transmitting infection in all stages of the life cycle except the egg. Rickettsiae have not been recovered from vertebrate animal reservoirs in some instances. Thus small rodents, rabbits and the larger goats and the opossum may harbor virulent organisms of spotted fever and the dog appears to be a reservoir of fièvre boutonneuse and Kenya fever.

## THE TYPHUS FEVER GROUP

Classical or louse-borne typhus fever has been known in Europe for many centuries and has repeatedly been present in devastating epidemics of high mortality. It was probably introduced into Mexico during the Spanish Conquest, where it has also been epidemic, and during the nineteenth and early twentieth centuries it was recognized in American seaports among immigrant populations (ill's disease). Endemic foci of classical typhus fever are recognized within the human population in Central Europe (Russia, Poland). The disease is particularly prevalent during the cold months of the year, in correspondence with louse infestation and a high rate of louse infestation. Epidemics are particularly associated with crowding, filth, wars and other causes of disrupted hygiene and sanitation. The fatality rate of epidemic typhus varies between 8 and 70 per cent of cases.

Murine, flea-borne or endemic typhus is, on the other hand, widespread in

distribution and is maintained in the rat population. Endemic typhus is present in the southern United States, Mexico (tabardillo), Malaya (urban or shop typhus), Europe, North Africa and Manchuria, and has been recognized elsewhere. The disease is transmitted among rats by fleas and rat lice and only sporadically does it affect man. The human body louse is, however, susceptible to rickettsial infection. Murine typhus and in a heavily infected population may contribute to the spread of the disease in man. The fatality of murine typhus is generally low.

In man, rickettsiae of classical and murine typhus give rise to identical symptoms of disease. After an incubation period of approximately twelve days the disease generally begins abruptly with malaise, fever, severe headache, vomiting, cough and, later, to stupor and delirium. Within a few days a reddish-purple, macular, papular or blotchy rash develops and persists until the disease subsides by crisis after about two weeks of illness. Murine typhus is generally a less severe disease.

Typhus rickettsiae are present in large numbers within the body louse, and this insect may transfer rickettsiae through bite or feces within a few days after infection. The louse succumbs to its infection in approximately twelve days. Fleas and the rat louse are thought to transmit rickettsiae of the endemic disease through the feces. The infection is not fatal to these insects.

#### THE TSUTSUGAMUSHI GROUP

The mite-borne fevers of the tsutsugamushi group are limited in geographical distribution to the Orient (Japan, Formosa, Burma, the Philippine Islands, Sumatra, and Malaya). In these areas the diseases occur particularly among rural populations, agricultural and plantation workers, who enter mite-infested areas and handle mite-infested wood and crops. The diseases are alike immunologically and in their transmission by the infected larval stages of mites of the genus *Trombicula* (*T. akamushi*, *T. deliensis*), the nymphal and adult stages of which are free-living and, hence, do not transmit the rickettsiae. In Japan tsutsugamushi disease (flood fever, river fever) occurs in scrub areas along large rivers which harbor the vole. The disease in Formosa and Sumatra (mite fever, mijtekoorts) is identical with that in Japan, except that the animal reservoirs are the field and house rats. In Malaya, infection with *R. tsutsugamushi*, known as rural or scrub typhus, is endemic in the rat in rural areas, where it is an important disease among agricultural workers. The diseases are more frequent in males than females and are fatal in approximately one-sixth to one-third of the cases. In Japan the infection is more frequent during the summer, corresponding to activity of the mites and to the flood season. In Malaya there is little seasonal variation.

Tsutsugamushi disease is an acute febrile disease beginning two to three weeks following the mite bite. The development of an initial papular lesion of the skin which progresses to a necrotic ulcer is typical of the disease, although this lesion is inconsistently present in mite fever of Sumatra and absent in scrub



us. A generalized maculo-papular skin rash appears during the second week of the disease. There is enlargement of the lymph nodes, malaise and signs of involvement of the central nervous system and respiratory system.

### Q FEVER GROUP

The Q fever group is unusual among rickettsial diseases in that the Weil-Felix reaction is negative and there is usually no skin rash. The disease, which was originally described from Australia, has now been identified in the western United States (Nine-mile fever), Panama, the Mediterranean area and the Balkans (Balkan grippé). Q fever occurs in two forms; the one is an acute fever without important localizing symptoms or findings, whereas the second closely resembles "virus" pneumonia. Although the exact mode of transmission to man and natural animal reservoirs are unknown, the former disease is apparently tick-borne and the second is presumably air-borne. *Rickettsia burneti* has been isolated from ticks and bandicoots in Australia. In the United States the causative agent has been identified in ticks collected near Nine Mile Creek in Montana. In man there appears to be an increased risk of infection among laboratory workers, foresters and packing and dairy employees in infected areas. In Panama identification of the human disease was made during studies of atypical pneumonia, and in Europe rather extensive outbreaks were encountered among soldiers and laboratory personnel in the Italian theater and the disease has been identified with Balkan grippé. Q fever was first recognized in 1935, but its incidence and geographical distribution are still incompletely known.

### RICKETTSIALPOX

A new disease, later found to be a rickettsial infection, was recognized in New York City in 1946 among residents of a housing development. The disease was a nonfatal febrile infection characterized by an initial and later generalized skin eruption which resembles that of chickenpox. The causative agent, *R. akari*, was recovered from the blood of patients, rodent mites and the common house mouse during this outbreak, so that it appears that the rodent and mites are the source of human infections. *Rickettsia akari* immunologically is somewhat related to the rickettsiae of spotted fever. Since this is the only break of rickettsialpox so far reported, the geographical distribution of the disease is unknown.

### CONTROL AND TREATMENT OF RICKETTSIAL DISEASES

Control of the rickettsial diseases is dependent upon eradication or avoidance of exposure to the arthropod vector, avoidance of heavily infested areas, control of the vertebrate reservoirs of infection and vaccination of human beings. Not all measures are equally effective in the several diseases. Thus, delousing methods

TABLE 16. THE HUN

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# NETTSIAL DISEASES

PATHOGENICITY		ARTHIPOD VECTOR	CHIEF GEOGRAPHICAL DISTRIBUTION
HUMAN DISEASES	ANIMAL RESERVOIR		
Eastern and Western Rocky Mountain spotted fever	Rabbits, wild rodents, mountain goats	Ticks: <i>Dermacentor andersoni</i> , <i>Dermacentor variabilis</i> , <i>Hemaphysalis leporispalustris</i>	United States (widespread)
Paulo typhus	Opossum, rabbit, dog	Ticks: <i>Amblyomma</i> spp.	South America: Colombia, Brazil
African tick fever		Ticks: <i>Amblyomma</i> , <i>Hemaphysalis</i> , <i>Boophilus</i> , <i>Rhipicephalus</i>	South Africa
Levantine boutonniere fever	Dog	Ticks: <i>Amblyomma</i> , <i>Rhipicephalus</i>	Mediterranean region, South Africa
Murine typhus fever (European typhus, louse-borne typhus) (formerly in U. S.)		Body louse: <i>Pediculus humanus</i>	Europe
Murine or murine typhus	Wild rats, other rodents	Rat flea and rat louse: <i>Xenopsylla cheopis</i> , <i>Polyplax spinulosus</i>	World-wide
Wugamushi disease (fever) (Sumatra) (or scrub typhus) (Malaya)	Field mouse (vole—Japan) Field rat (Sumatra) Malayan rats	Mites: <i>Trombicula akamushi</i> and other species	Japan, Sumatra, Malaya
Queensland and American scrub typhus (nine-mile fever) (in grippe)	Bandicoot in Australia	Ticks: <i>Dermacentor</i> spp., <i>Hemaphysalis</i> sp., <i>Amblyomma</i> sp.	Australia, United States, Panama, Europe
Rocky Mountain fever	Unknown	Body louse: <i>Pediculus humanus</i>	Europe
Rocky Mountain spotted fever	House mouse	?Mite: <i>Allodermanyssus sanguineus</i>	Recently described in New York City

have proved successful in control of classical typhus fever and vaccination been of some value, particularly in the murine disease. In spotted fever persons protective measures and vaccination have been most successful, whereas habits of the vector ticks render control measures less successful. In tsutsugamushi diseases, eradication of brush and domestic harborage of rodents may be of some value.

Treatment of the rickettsial diseases is relatively unsatisfactory. Immune sera have had some experimental trials with somewhat encouraging results in the case of spotted fever. Chemotherapy and antibiotic therapy with penicillin and streptomycin are unsatisfactory. However, para-aminobenzoic acid experimentally inhibits growth of rickettsiae in the chick embryo and has therapeutic value in human infection. Current trials with the antibiotic chloromycetin are promising and aureomycin has been used successfully.



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## VIRUS DISEASES

The animal viruses, including those which infect man, are a heterogeneous group known by their pathogenic effects and immunology. The nature of these viruses has been discussed in Chapter 8, so that it is necessary to say only that they have in common the characteristic of **cytotropism**; they are obligate intracellular parasites. The viruses and rickettsiae, which are also obligate intracellular parasites, have recently been classified according to the accepted rules of nomenclature. This classification is in part presented in the accompanying table. The rickettsiae and Bartonellaceae have been discussed previously. However, the Myxozoonaceae of the order Rickettsiales consists of agents commonly included with the viruses. The order **Virales** as presently constituted has three members, the **Phagineae** (bacteriophages), **Phytophagineae** (plant viruses) and **Zoophagineae** (animal viruses). Agents of human disease are, of course, included in the Zoophagineae. As a group the viruses infect all tissues of the body. However, many are localized or produce their major effects in particular tissues. Thus, the **dermotropic** and related viruses affect particularly the skin; the **pneumotropic** group produces its sole or major effects in the respiratory tract; the **viscerotropic** viruses may be generally distributed but produce infection of the deep tissues and organs; and the **neurotropic** agents are injurious to the central nervous system.

The tissue damage produced by viral infection may be either primarily destructive and inflammatory or proliferative; for example, warts and viral diseases of animals. Except for a few infections, notably the protozoal disease malaria, the viruses are unique in their intracellular parasitism. This location undoubtedly influences the pathology and immunity in virus infections. Thus the virus may multiply and destroy the cell or stimulate it to multiplication without producing relatively little inflammatory reaction. The effects are in many ways analogous to those of naturally occurring enzymes. Furthermore, the intracellular position protects the virus against extracellular antibodies and cellular defense mechanisms. Bacterial toxins after adsorption to the cell are similarly protected from the action of antitoxin.

Immunity in virus diseases does not differ materially from antibacterial immunity. In some infections, *e.g.*, measles, acquired resistance is highly effective and enduring, whereas in others, *e.g.*, influenza and herpes simplex, repeated infections are common. Antibodies exert protection against the specific virus in

TABLE 17. CLASSIFICATION OF ANIMAL VIRUSES AND RELATED ORGANISMS \*

ORDER	FAMILY	GENUS AND SPECIES	DISEASE
Rickettsiales	Rickettsiaceae	<i>Rickettsia</i> spp. }	Rickettsial diseases
		<i>Coxiella</i> sp. }	(see Chapter 39)
	Bartonellaceae	<i>Bartonella bacilliformis</i>	Bartonellosis
			(see Chapter 37)
	Chlamydozoaceae	<i>Chlamydozoon trachomatis</i>	Trachoma
		" <i>oculogenitale</i>	Inclusion conjunctivitis
		<i>Miyagawanella lymphogranulomatis</i>	Lymphogranuloma venereum
		" <i>psittacii</i>	Psittacosis
		" <i>ornithosis</i>	Ornithosis
		" <i>pneumoniae</i>	Pneumonitis of man
" <i>louisianae</i>		" " "	
Virales Suborder Zoophagineae		" <i>illinii</i>	" " "
		" <i>bronchopneumoniae</i>	Pneumonitis of lower animals
		" <i>felis</i>	
	Borreliotaceae	<i>Borreliota variolae</i>	Smallpox (variola), vaccinia and alastrim
	Pox viruses and related forms	<i>Briareus varicellae</i>	Chickenpox and herpes zoster
		" <i>morbillorum</i>	Measles
		<i>Scelus recurrens</i>	Herpes simplex or febril
		" <i>suillum</i>	Animal viruses: pseudo
		" <i>marmorans</i>	rabies in swine, ectron
		" <i>bovinum</i>	of mice, bovine stoma
" <i>ulceris</i>		and ovine balano-post resp.	
		<i>Molitor verrucae</i>	Human warts (verruca)
		" <i>hominis</i>	Molluscum contagiosum
		" <i>tumoris</i>	Fowl sarcoma
Erronaceae Encephalitis group		" <i>sylvilagi</i>	Rabbit papilloma
		" <i>myxomae</i>	Rabbit myxoma
	<i>Erro scoticus</i>	Louping ill	
	" <i>silvestris</i>	Spring-summer encephal	
	" <i>incognitus</i>	X disease	
	" <i>japonicus</i>	Japanese B encephalitis	
	" <i>nili</i>	West Nile encephalitis	
	" <i>salestus</i>	St. Louis encephalitis	
	" <i>equinus</i>	Equine encephalomyelitis	
		<i>Legio debilitans</i>	Poliomyelitis
Charonaceae		" <i>erebea</i>	Lymphocytic choriomen
		" <i>muris</i>	gitis
		<i>Formido inexorabilis</i>	Theiler's mouse poliomye
		<i>Charon evagatus</i>	Rabies
		" <i>vallis</i>	Yellow fever
		<i>Tarpeia alpha</i>	Rift Valley fever
		" <i>beta</i>	Human influenza
		" <i>premians</i>	" "
		" <i>canis</i>	Common cold
		<i>Tortor suis</i>	Canine distemper
Rabulaceae		" <i>bovis</i>	Hog cholera
		<i>Rabula inflans</i>	Rinderpest of cattle
Borrelomyceta-ceae		" spp.	Mumps
		<i>Asterococcus</i>	Animal salivary gland viruses
			Pleuropneumonia group

\* Incomplete list from Bergey, *Manual of Determinative Bacteriology*, Ed. 6, 1948. Williams & Wilkins Co., Baltimore.



and animals and in addition may often be demonstrated by the complementation reaction or the precipitation and agglutination of virus particles. In recent years the inhibition by specific immune serum of the agglutination of human erythrocytes by certain viruses has been of particular interest and of great value as a test for virus and its antibody.

For present purposes the viral diseases will be discussed as dermatropic, viscerotropic and neurotropic groups. It should be realized by the student that the virus is not necessarily restricted within these locations, but rather that the major pathology is produced in these tissues. The psittacosis-typhus group is discussed separately since these agents affect other tissues but are otherwise related.

### DERMOTROPIC VIRUSES

**Smallpox—Variola and Vaccinia.** Smallpox (variola) is an ancient and, in the absence of effective control, a currently important disease. The origin of smallpox is undecided, but it may have been Africa or Asia. It was known in India by the third century B.C. and had been introduced into Southern Europe during the medieval era. It was recognized in England by the sixteenth century. Throughout its history smallpox has occurred in epidemics of greater or lesser virulence and has been greatly feared as a cause of death, disfiguring, scarring and blindness. Smallpox is also of historical interest in that it was the first disease against which preventive immunization was found to be effective. The ancient practice of **variolation**, that is, the inoculation of smallpox virus as a preventive measure against the naturally occurring disease, was replaced by the remarkably effective and more innocuous procedure of **vaccination** with cowpox virus as described by Edward Jenner in 1795. Vaccination against smallpox was introduced into the United States by Benjamin Waterhouse in 1800 and remains a widespread but not universal practice.

Smallpox is a generalized viral infection, the characteristic lesions of which occur on the skin and mucous membranes, including the conjunctivae. The typical skin lesions begin as red macules and papules which within a few days develop into fluid-containing vesicles, then pustules followed by crust formation and healing in approximately three weeks. Generalized symptoms of malaise and fever may be severe. In the usual form of the disease the lesions are single, although severe confluent and hemorrhagic types and mild infection in vaccinated individuals (varioid) may occur. In addition to the severe disease resulting from infection of susceptible individuals with classical variola virus, characteristically benign or mild disease caused by the less virulent **alastrim** virus has been identified. Mortality of classical smallpox may be 30 per cent or higher, whereas the fatality of **alastrim** is generally less than 1 per cent. **Vaccinia**, which is the infection following preventive vaccination with modified virus (see above), generally produces one or a few typical pox at the site of inoculation. The virus is present in the skin lesions of variola, **alastrim** and **vaccinia** and in the

respiratory secretions in the natural disease. Infection may thus be acquired either by droplets or by direct contamination from the fluid or crusts of the lesions. Vaccinia is not contagious but contact transmission may occur.

**The Virus (*Borreliota variolae*).** The virus of smallpox is among the larger filtrable viruses. It is generally distributed in the body in both variola and vaccinia. Examination of infected tissues under the microscope reveals large inclusion bodies (Guarnieri bodies) within the cytoplasm of infected cells. These bodies, originally described as protozoan parasites, are now recognized as viral colonies which contain large numbers of smaller elementary particles of infectious virus.

Study of the properties of *Borreliota* has been made largely with vaccinia virus, which may be purified by ultracentrifugation. The size of the virus is determined by ultracentrifugation and measurement of electron micrographs; it is variable but is approximately 225 m $\mu$ . From physicochemical studies the particles appear to have a density of 1.16 in aqueous suspension and to be composed of protein, lipids and the desoxypentose type nucleic acid. Enzymatic activity of the elementary particles has not been unequivocally established.

Immunological studies have indicated the complexity of the antigenic material of vaccinia and the separation of heat stable and heat labile fractions. There are certain differences in virulence, morphology of inclusion bodies and cross-protection between the viruses of variola, alastrim and vaccinia. The relationship between these viruses is, however, a close one. Thus, variola virus from human lesions when passed in rabbits and calves is modified in virulence and immunology to vaccinia virus. These derived strains are for practical purposes identical with natural cowpox virus, and vaccinia virus from either source may be used for vaccine production.

**Epidemiology.** Following its introduction into Western Europe smallpox became one of the reigning communicable diseases. It may affect all age groups and at one time or another has occurred primarily in adults or children, reflecting the relative immunity of these groups. It is a disease of contact and is most prevalent in the winter. At the present time smallpox prevalence appears definitely to be related to the degree of immunization of the population.

The variation in severity of epidemics of smallpox has been noted since early times, and currently the mild alastrim or *variola minor* appears to be more prevalent than the classical disease or *variola major*. These diseases which appear to be etiologically distinct may occur in the same locality, and the increased frequency of alastrim, while it reduces the mortality of smallpox, increases the difficulties of control through failure to recognize the infection as smallpox. The control of smallpox depends upon diagnosis and isolation of cases and extension of vaccination within the population. Several hundreds of thousands of cases occur annually within the United States and the disease is occasionally introduced into the large cities. In general, however, the prevalence is low and epidemics are of small size.



**Immunity.** Recovery from smallpox results in prolonged immunity against second infection, and, of great practical importance, vaccinia produced by inoculation of vaccine virus confers protection against smallpox in man. The duration of protection appears to be at least five years and may be considerably longer.

Vaccine for human vaccination generally is produced in calves (calf lymph), although tissue culture and chick embryo virus may be used. Healthy calves are inoculated in scratches in the shaved, carefully cleansed skin of the abdomen and at the height of the resulting vaccinia, the calf is sacrificed and the lesions are harvested. The resulting "lymph" is suspended in glycerin solution, carefully standardized for potency, tested for bacterial content and dispensed in individual vials. In human or jennerian vaccination the contents of one vial are dropped onto the cleansed skin and are inoculated into the superficial epidermis by multiple pressure with a sharp needle, after which excess vaccine is wiped off. The skin is not penetrated in this method of vaccination as in the older scratch method.

The reaction to vaccination may be either development of a complete "take," or, vaccinia, an accelerated or an immune reaction. In the first, a typical pock develops at the site of inoculation and requires approximately 21 days for complete evolution and healing. In the accelerated reaction the lesion develops only partially or evolves at a more rapid tempo, whereas in the immune reaction only a transitory reddened papule appears in approximately 48 hours after inoculation.

It is usually recommended that vaccination be performed in infants during the second six months of life and on children at the beginning of school attendance. In the absence of smallpox in the locality, the above procedure usually provides adequate protection. When smallpox is present, however, vaccination of the population should be performed irrespective of past history.

Both variola and vaccinia result in the formation of specific neutralizing, complement-fixing and precipitating antibodies against both types of virus.

**Animal Pock Diseases.** Virus disease characterized by cutaneous lesions similar to those of variola, vaccinia and varicella in man occur in many lower animals, including horses, sheep, swine and fowl. The viruses of these diseases appear to be separate entities but to have some relationship to the human pock diseases. The evidence suggests an evolutionary relationship between the pock viruses.

**Chickenpox (Varicella).** Chickenpox is a highly communicable and prevalent disease of childhood, the lesions of which resemble those of variola. They are, however, more superficial, produce less scarring and differ in their distribution over the body. Intranuclear and sometimes intracytoplasmic inclusion bodies are present within the vascular endothelium and cutaneous epithelium of the lesions. Although varicella is communicable to man, laboratory study of the etiological agent and infection of experimental animals have been difficult. Varicella is generally not a severe disease, although complications, the most

serious of which is involvement of the central nervous system, sometimes of the eye. Complement-fixation and agglutination have been demonstrated with immune serum and elementary bodies in the vesicle fluid. Recovery is associated with resistance to reinfection.

There appears to be an epidemiological and immunological relationship between herpes zoster (shingles) and varicella, although the evidence is not complete. The former disease is characterized by the presence of vesicular skin lesions, which contain elementary bodies, along the distribution of one or more cutaneous nerves.

**Herpes Simplex.** The most common form of herpes simplex (herpes febrilis) is the well known "fever blister" or "cold sore" of the lips and nose. Infection may, however, occur on other areas of the skin and mucous membranes and has been related to the more serious aphthous stomatitis of children. Herpes affects a high proportion of the population in all geographical regions of the world. The disease is not highly communicable but infection appears to be acquired by contact and, once established, to persist in the same host indefinitely with repeated recurrences. Although the disease is ordinarily not serious, involvement of the central nervous system with encephalitis may occur and host-parasite relations are particularly interesting. Infection is associated with the development of neutralizing and complement-fixing antibodies. The antibody titer is generally high or completely negative, a finding presumably accounted for by the persistence of virus within the body.

The virus of herpes is one of the larger viruses, 100–150  $m\mu$  in diameter. Vesicle fluid is infectious and reportedly contains elementary bodies. Inclusion bodies have been described from both the nucleus and cytoplasm of infected cells. The most characteristic finding is the eosinophilic granular intranuclear inclusion or Lipschütz body. Herpes virus is infectious for rabbits by the intracorneal, intracutaneous and intracerebral routes, giving rise to typical local lesions which contain inclusion bodies, and to fatal encephalitis. Infection may also be produced in other laboratory animals, and the virus may be propagated in the chick embryo and in tissue cultures.

**Molluscum Contagiosum.** Molluscum contagiosum is a small benign cutaneous viral tumor of man. The lesions consist of small areas of proliferation of the epidermis with a central body of degenerated cells (a few millimeters in diameter). Infected cells contain large intracytoplasmic inclusion bodies made up of elementary bodies. Molluscum is transmitted by direct contact. The lesions generally disappear spontaneously, but they may be spread to other areas of skin by autoinoculation.

**Warts and Viral Tumors.** Human warts (verrucae) and a number of tumors of animals are transmissible by filtrates of infected tissues. Human warts are autoinoculable and transmissible to man but not to lower animals. There are no specific inclusion bodies within the epithelial cells of the irregular growths. Filtrable epithelial tumors, papillomas, occur in several lower mammals and at least one, the rabbit papilloma, is subject to malignant degeneration.



is, cancer formation. In addition, certain tumors of connective or lymphoid elements of lower animals appear to be of viral etiology.

**Foot and Mouth Disease.** Foot and mouth disease is a highly fatal and economically important viral disease of cattle. The disease is transmissible from infected animals to man, presumably by contact or contaminated milk and milk products. The lesions consist of virus-containing vesicles of the mucous membranes and skin. Human infection is infrequent and secondary to the disease in animals.

**Trachoma, Inclusion Conjunctivitis, Epidemic Keratoconjunctivitis.** Trachoma is a chronic infection of the conjunctiva and cornea with vascularization or pannus formation and later scarring. Inclusion bodies, which are morphologically similar to those of the psittacosis group and the rickettsiae, are characteristic of trachoma. Indeed, the etiological agents of both trachoma (*Chlamydozoon trachomatis*) and inclusion conjunctivitis (*C. oculogenitale*) are presently classified with the Rickettsiales.

Trachoma is an ancient disease present in many parts of the world. In the United States its distribution is geographically limited to parts of Arkansas, Missouri, Illinois, Kentucky, West Virginia, Tennessee and to Indian reservations. The disease affects all age groups, both sexes and all racial groups. Transmission appears to be favored by close personal contact under poor hygienic conditions. The infection may be transferred experimentally in animals by means of cell-free filtrates. The viral nature of trachoma is now recognized, although in the past many bacteria, chief of which is the *Bacterium granulosis* of Noguchi, have been associated with the disease.

Several types of conjunctivitis have some similarities to trachoma. Thus inclusion blennorrhea or inclusion conjunctivitis of the newborn is caused by *C. oculogenitale*. This disease, which appears to be acquired by congenital transmission, lasts for several weeks or longer and does not result in scar formation. Inclusions similar to those of trachoma are present within the infected cells. In both diseases appear to respond to drugs of the sulfonamide series. Swimming-pool conjunctivitis of the adult is likewise a benign conjunctivitis, which has inclusion bodies within the epithelial cells.

Epidemic keratoconjunctivitis was first recognized in the United States in 1941. The disease has, however, been recognized previously elsewhere. The disease is an acute, highly infectious conjunctivitis from which a virus has been identified by animal experimentation, and antibodies have been demonstrated in convalescent serum. The disease occurs in epidemic form and appears to be transmitted by contact.

**Measles (Morbilli, Rubeola).** Excepting the common cold, measles is probably the most common acute communicable disease of man. It affects children primarily, but adults who have escaped previous infection are susceptible. Prolonged immunity, thus, appears to result from clinical or possibly subclinical infection. Susceptibility is naturally high and this fact together with the great communicability of the disease leads to almost universal clinical attack.

Convalescence confers a high degree of immunity. Furthermore, there is still lived congenital passive immunity in infants of immune mothers. With the extinction of certain isolated communities into which the disease may be occasionally introduced, measles is world-wide in distribution. Several hundred thousand cases, chiefly in children, are reported annually in the United States. It is, however, an important cause of death only in the very young age group, in which secondary pneumonia may be a complication. The epidemiology of measles has been extensively studied, particularly with regard to the periodicity of epidemics. Increased prevalence tends to recur every two to three years in many areas although the periodicity varies in different localities. The disease is most frequent in the winter and spring.

The acute symptoms of measles begin after an incubation period of approximately two weeks and are characterized initially by fever, coryza, cough and conjunctivitis. Within a few days typical small white patches (Koplik's spots or measles enanthem) appear on the mucous membrane of the mouth and throat and the dull red, blotchy (morbilliform) skin rash or exanthem develops. The disease is transferred by respiratory droplets before and during the early eruptive stage.

Available evidence indicates that measles is a viral infection. The agent is filtrable and is present in the nasopharyngeal secretions and, in the early stages, in the blood of human cases. It may be transferred to man and to monkeys and cultivated in chick embryos.

Prevention and control of measles by epidemiological methods is notably unsuccessful. The disease is difficult to recognize during the highly infectious pre-eruptive stages, and there is no accepted method of vaccination. However, passive immunization is successful and in normal individuals other than very young infants it is probably best used for modification rather than for complete prevention of the disease. Human convalescent serum and concentrated immunoglobulin are given in doses of 2 to 10 ml. during the incubation period. The mild, modified disease following use of serum late in the incubation period provides active immunity against subsequent attack while the severe infection is prevented. Complete prevention of the disease may be obtained by the early administration of serum or globulin.

*Rubella (Three-day Measles, German Measles).* Rubella or true measles does not confer immunity against rubella, which appears to be a separate communicable disease of viral etiology. Rubella is communicable and usually observed in epidemics. The disease is in general not serious, although infection of the mother during early pregnancy appears to be correlated statistically with the occurrence of certain congenital defects in the infant.

#### VISCEROTROPIC AND SYSTEMIC VIRUSES

**Yellow Fever.** Yellow fever is an acute febrile disease characterized by jaundice, midzonal necrosis of the liver and metabolic disturbances resulting



a malfunction of the liver. The disease presumably had its origin in Africa and, with the exception of the Orient, has become widespread in tropical and subtropical regions of the world, apparently as the result of transfer by sea and air along the trade routes. Yellow fever is currently endemic in West Africa and South America. Widespread and highly fatal epidemics have occurred within the endemic areas and on the American continent as far north as the major Atlantic seaports of the United States and the inland cities of the lower Mississippi Valley. Control measures have eliminated yellow fever from the United States and now effectively control the epidemic disease in major cities elsewhere. However, present-day rapid transportation by air poses the problem of prevention of transfer of infection from endemic areas by this means.

**Transmission and Etiology.** Although suspected and incompletely demonstrated by Finlay, the transmission of yellow fever by the *Stegomyia* mosquito (*Aedes aegypti*) was proved by the United States Army Commission to Cuba (1900 and later). The Commission members (Reed, Carroll, Agramonte and Henshaw) were able to show in human volunteers that the agent of the disease is filterable, is present in the blood of yellow fever patients for a brief time early in the disease and is naturally transferred only by the bite of the infected female *Aedes aegypti*. This information provided the basis for the successful elimination of yellow fever from Havana by General Gorgas and later from the United States. However, the virus of yellow fever (*Charon evagatus*) was not established in animals until 1928 when Stokes, Bauer and Hudson reported infection of the *rhesus* monkey. Thereafter the virus was found infectious for man and certain other rodents by intracerebral inoculation. In contrast to the monkey, in whom the disease is similar to the human infection, mice develop encephalitis, and the virus is modified by repeated passage in these animals to a neurotropic virus, no longer capable of producing the typical disease in monkeys and man. The neurotropic virus is, however, antigenically the same as the unmodified viscerotropic strains. Neurotropic yellow fever virus may be further attenuated by tissue culture and is infectious for the chick embryo.

**Immunity.** Convalescence from yellow fever is associated with prolonged resistance to subsequent infection and the presence of neutralizing antibodies in the blood. For practical purposes immunity is lifelong in duration. Vaccine produced from chick embryos infected with neurotropic virus attenuated in tissue culture has been found effective against the disease in man and monkeys. The immunity following vaccination persists several years and perhaps longer. The extensive use of vaccination in endemic areas and in persons otherwise exposed to infection shows the value of vaccination in control of yellow fever.

**Newer Epidemiology and Control.** Following the demonstration that the female *Aedes aegypti* mosquitoes transmitted yellow fever and that mosquitoes remained infected throughout life, control measures directed against the mosquito (see Chapter 45) promised to eliminate the disease. Yellow fever control based on the "key center" hypothesis of eradication of *Aedes* from infected urban areas, introduced by General Gorgas, was brilliantly successful in Cuba, the

United States, Central America and South America. Yellow fever appeared to be all but eliminated when in 1928 and thereafter epidemics were reported in small towns and rural areas of Brazil. This disease, now known as jungle yellow fever, occurs sporadically in forest or jungle areas of Brazil and elsewhere. The disease occurs in the absence of *Aedes aegypti*, and other naturally infected culicine mosquitoes of the genera *Aedes* and *Haemagogus* have been identified. The lower mammalian reservoirs of jungle yellow fever appear to be jungle primates (monkeys).

The extent of human infection may be determined by serological studies of the blood for antibodies and the histological examination of specimens of liver from persons dying of acute febrile disease suspected of being yellow fever. Such examinations have been highly important in the study of the epidemiology of yellow fever in endemic areas.

It is thus apparent that the earlier epidemiological concept of yellow fever as a disease of man transmitted by *Aedes aegypti* represents only one part of the total picture and permits control of the urban epidemic disease but not of the jungle or rural type. The extensive jungle areas and the existence of lower animal hosts make eradication of yellow fever from these endemic rural foci highly improbable. Protection of the human population is afforded by vaccination and vigilance to prevent transportation of the disease to currently uninfected areas.

**Infectious Hepatitis.** An acute febrile disease characterized by liver damage and jaundice has long been recognized in many parts of the world as an epidemic disease among military personnel and presumably occurs as an institutional and childhood disease and as sporadic infection of the civilian population. The epidemic disease is recognized as infectious, presumably of viral etiology, and convalescence appears to be associated with specific resistance to reinfection. Experiments have demonstrated the infection of human volunteers following the feeding of infectious materials. The agent appears to be filtrable and present in the stools and blood of patients. The relationship, if any, of epidemic infectious hepatitis to catarrhal jaundice has not been established. The disease is separate from Weil's disease and yellow fever, and appears to be related to homologous serum jaundice and to jaundice following vaccination against yellow fever, which is observed particularly when immune serum is included in the vaccine.

**Dengue Fever.** Dengue fever (breakbone fever) is a noncontagious acute febrile disease the symptoms of which include severe pain in the joints, back, limbs and head and frequently an erythematous skin rash. The disease is endemic and sometimes epidemic in tropical and subtropical regions and is transmitted by *Aedes* mosquitoes (*Aedes aegypti*, *Aedes albopictus*). Human beings and monkeys from endemic areas are reportedly immune. The virus of dengue fever is infectious for monkeys, has been adapted to mice by intracerebral inoculation and may be cultivated in the chick embryo. The disease generally is of short duration and nonfatal, although repeated attacks may occur. Vaccination with mouse-adapted virus results in immunity in human volunteers.



**Rift Valley Fever.** Rift Valley fever of Africa is a disease of sheep transmissible to man. The disease in man is an acute febrile disease which occurs usually in shepherds in the enzootic area. In sheep the disease produces a striking degree of liver necrosis and is highly fatal. The virus is of small size and resembles that of yellow fever in the production of liver pathology. There is, however, no cross-immunity between Rift Valley fever and yellow fever, although the interference phenomenon may be demonstrated in experimental animals, in that yellow fever virus protects against simultaneously inoculated Rift Valley fever virus. An attack of Rift Valley fever confers lasting immunity.

**Phlebotomus Fever** (*Pappataci Fever*, *Sandfly Fever*). Phlebotomus fever is an acute viral disease of the tropics and subtropics which is transmitted by the sandfly *Phlebotomus papatasi*. Generalized aching and malaise, fever, nausea, headache and weakness begin suddenly and continue for only a short time. The causative virus is present in the blood early in the disease, at which time sandflies may be infected. The disease is prevalent during the hot dry months of the year in correspondence with the activity of the sandfly vectors. Recovery is associated with immunity to reinfection.

**Epidemic Parotitis** (Mumps). Mumps is a common communicable disease of man which attacks primarily children but which may also affect adults. Epidemics are observed among children and military personnel. The disease is most prevalent during the winter months in temperate zones. The virus of mumps attacks particularly the parotid glands, although the other salivary glands, the testes (mumps orchitis), the ovaries (oophoritis) and other glands may be affected. Monkeys develop parotitis following inoculation of mumps virus into the sublingual duct and the virus has been cultivated in the chick embryo, from which source suspensions of virus suitable for the preparation of vaccines and for the complement-fixation and skin tests may be obtained. Following vaccination of monkeys are reported to develop neutralizing and complement-fixing antibodies and to resist inoculation of active virus. In man an attack of mumps provides protection against subsequent infection and convalescence is associated with the presence of complement-fixing antibodies in the serum. A specific skin reaction to the intradermal inoculation of virus has also been described.

**Colorado Tick Fever.** Colorado tick fever is an acute relapsing fever which occurs during the warm months of the year in the Rocky Mountain region, particularly Colorado. The virus appears to be of very small size and is infectious to hamsters, mice and the developing chick embryo. The wood tick *Dermacentor andersoni* has been incriminated as the vector on both epidemiological and laboratory evidence.

## THE PNEUMOTROPIC AND RELATED VIRUSES

**Influenza.** Epidemics and pandemics of clinical influenza have occurred periodically since the sixteenth century and lesser outbreaks of a clinically similar but less fatal infection are observed at frequent intervals. In extensive outbreaks,

the last of which occurred in 1918-1919, as many as one-fourth to one-half the population may be infected and the mortality from the disease and its complications may be exceptionally high. All age groups, both sexes and all races of man are naturally susceptible to the disease. Transmission is by droplet and air-borne infection, and epidemics tend to be explosive in character with several recurrent waves of increased prevalence (see Chapter 44). Clinically influenza is an acute upper respiratory infection with coryza, fever and severe general malaise, aching, weakness and malaise. Involvement of the lungs with secondary pneumonia is frequent and accounts for a high percentage of fatalities.

**Etiology.** Many types of bacteria including most of the flora of the nose and throat have been associated with influenza. Of particular importance is the *Hemophilus influenzae* described by Pfeiffer in 1892 in association with the great pandemic of 1889-1890. Until investigations during and after the 1918-1919 pandemic demonstrated that its presence was inconsistent and its role not primary, *H. influenzae* was generally regarded as the causative agent of human influenza. The primary viral etiology of the disease was demonstrated first in the related swine influenza by Shope (1930) and in human influenza by Smith, Andrewes and Laidlaw (1933).

**Swine Influenza.** Swine influenza, first recognized in 1918, is prevalent during the fall and winter months in the middlewestern United States. The typical disease has been found to follow infection with the virus of swine influenza together with a bacterium, *Hemophilus influenzae suis*. The virus in the absence of the bacterium induced only a mild disease. Swine influenza virus is immunologically related to human influenza virus Type A. Epidemiology of the disease is particularly interesting in that the virus appears to be transferred in the ova of the swine lungworms, which may contain virus. The ova are ingested by earthworms, in which they develop into infectious larval forms, and the swine acquire infection by eating the earthworms. Infected pigs may then develop clinical influenza following some adverse stimulus.

**Human Influenza.** Human influenza may be reproduced by inoculation of the virus alone. Two immunologically distinct types of human influenza virus, Types A and B, as well as subtypes, are recognized at the present time. These viruses are approximately 70 to 120 m $\mu$  in diameter and appear to be antigenically complex. The results of chemical analysis have been variable, but they indicate the presence of nucleic acids, lipids and a relatively large amount of carbohydrate. Human influenza viruses are infectious for ferrets, mice, swine and less regularly other animals, and are readily cultivated in the allantoic cavity of the chick embryo. Chicken red blood cells are agglutinated in the presence of influenza virus (Hirst phenomenon) and the agglutination is specifically inhibited by immune serum against the virus. The identification of two immunologically different types of virus, both of which give rise to typical influenza, has helped to clarify the epidemiology and immunology of the disease. Thus, the different types of virus are prevalent within the population periodically.



independently, so that epidemics may be related to either Type A or Type B virus, or both may be present simultaneously.

**Immunity.** Recurrent attacks of influenza are commonly observed, and immunity against one type of virus does not protect against infection with another type. An attack of influenza results in a sharp rise in specific serum titer against the causative virus. The rise of titer is, however, transitory, consistent with this finding is the apparent short duration of resistance to infection. Immune serum contains both neutralizing or protective and complement-fixing antibodies and inhibits the agglutination of chicken erythrocytes by strains of virus which are immunologically identical or very closely related to that originally described by Smith, Andrewes and Laidlaw are included in Type A, whereas antigenically different viruses are included in Type B.

Active immunization against influenza, *i.e.*, vaccination, has been found to protect both man and animals against infection with the same type of virus. In large-scale trials of active immunization in man the incidence of clinical influenza has been found lower in the vaccinated groups, so that vaccination can be recommended as a preventive procedure. It must be recognized that protection is not conferred against immunologically different types of virus. Vaccines currently in use consist of a formalinized suspension of mixed Types A and B viruses from chick embryos to which adjuvants may be added in order to increase the immunizing power of the virus. Immune serum, while it has not been widely used in human influenza, experimentally protects animals against infection.

**The Common Cold.** Acute upper respiratory infection, chiefly the common cold, is the most frequent cause of illness and disability within the population. The etiology of the cold, characterized by nasal congestion, coryza, enlargement of the lymphoid tissues of the pharyngeal region, mild fever and malaise, has been the subject of many investigations. The disease appears to be communicable, and the acquisition of an infectious agent the primary factor in the development of symptoms. It is undecided whether a cold is a specific disease entity with a specific causative agent or the result of infection with any of several pathogens. Sometimes, certain bacterial infections of the upper respiratory passages may give rise to symptoms of a cold as may certain specific communicable diseases. The common cold, however, is not closely correlated with bacterial infection, and no causative agents have been isolated by a number of investigators. Dochez and his co-workers, and recently Topping and their collaborators have been able to reproduce the symptoms of the common cold with bacteria-free materials and have propagated the agent in chick embryos. The agent would thus appear to be a filtrable virus, though little is known of its nature, or whether one or several agents may be responsible for the infection. Immunity is apparently of short duration and recurrent attacks, often within a few months, are the rule.

**Primary Atypical Pneumonia.** Within the past ten years sporadic and epidemic infections of the lungs have been widely recognized. The disease is a primary atypical pneumonia or pneumonia with patchy infiltration of the lungs, fever, malaise

and cough, which except for the primary involvement of the lungs is not unlike influenza. The infection appears to involve the smaller bronchial structures and the adjacent lung tissue. Essentially similar disease has been related to infection with several different viruses and with *Rickettsia burneti*. Prominent among viral agents are several members of the psittacosis group, although as yet incompletely described viral agents have been isolated. In infections with the latter agents the development of cold agglutinins ( $4^{\circ}\text{C}$ ) for human erythrocytes is of diagnostic importance. Atypical pneumonia has been epidemic among students and military personnel in whom it has assumed importance as a cause of disability. Recovery is the rule. The course of the disease is not modified by sulfonamide drugs or penicillin, but in many instances aureomycin is an effective antibiotic and chloromycetin may be of value.

### THE NEUROTROPIC VIRUSES

**Rabies.** Rabies (hydrophobia) is a viral disease of lower mammals, particularly the carnivores (dogs, cats and wolves), transmissible to man. This disease has been recognized since antiquity and in its more recent history is interesting in the discovery of methods for active immunization by Pasteur and for diagnosis in animals by demonstration of inclusion bodies (Negri). Together these discoveries have provided the basis for the protection of man and animals from this dread and highly fatal disease.

Human rabies is contracted from infected animals, generally from a bite, but occasionally from contamination of skin lacerations with saliva. The incubation period varies from a few weeks to several months and is relatively short when infection occurs about the head, neck and upper extremity. The symptoms and lesions of rabies are the result of infection of the central nervous system and are somewhat variable. Muscular spasms and paralyses, including the muscles of swallowing, attacks of fury and delirium with the characteristic "fear" of water (hydrophobia) are the major symptoms. The lesions of the nervous tissue are both degenerative and inflammatory and contain Negri bodies. The pathogenesis of rabies is particularly interesting in that following its introduction into a bite or laceration the virus has been found to migrate along the peripheral nerves and to produce symptoms following its establishment in the central nervous system. The disease in carnivores is essentially similar to that in man in that the symptoms are due to infection of the nervous system. Extreme irritability, changes in behavior, salivation associated with inability to swallow, followed by paralyses, stupor and death are the usual symptoms. The incubation period of rabies in the dog is also variable, but the fact that rabies virus is demonstrable in the saliva for only a few days prior to the onset of symptoms is highly significant to the prevention of rabies in man.

**Rabies Virus.** Rabies virus (*Formido inexorabilis*) is relatively large in size (100–150 m $\mu$ ) and different strains appear to be immunologically homogeneous. The virus is not unusually resistant to deleterious agents and may be



ivated in the chick embryo and in tissue culture. The typical acidophilic inclusion or Negri bodies are generally but not universally present in the cytoplasm of infected cells, although smaller elementary bodies have not been demonstrated. The virus is infectious for a large number of domestic, wild and laboratory animals, and is found in the nervous tissue, the salivary glands, saliva and sometimes other tissues and secretions. The strains of the natural virus ("Street virus" of Pasteur) vary considerably in virulence for laboratory animals. When a virus is serially inoculated into rabbits by the intracranial (subdural) route its virulence becomes markedly increased for direct inoculation of the nervous system and, of great significance, it is reduced following inoculation by other routes. (Such "fixed virus" strains are used for production of rabies vaccines.)

**Diagnosis.** The diagnosis of human rabies is a clinical or postmortem one. Laboratory diagnosis of infection in animals is accomplished by demonstration of Negri bodies or of the virus. Negri bodies are most numerous in the hippocampus (Ammon's horn) and in the cerebellum of infected brains. Emulsion smears from these areas, stained by any of several methods, reveal the Negri bodies within the cytoplasm of the cells. In the absence of Negri bodies, infection may be demonstrated by inoculation of animals, preferably mice, although guinea pigs and guinea pigs are also susceptible. These animals become ill and die within two weeks following inoculation with emulsion of infected tissues. Since recognition of rabies in animals, particularly in dogs, is of primary importance to the protection of man, every precaution should be taken to permit accurate diagnosis.

Whenever possible a dog suspected of being rabid should be confined under observation for a period of two weeks. Since virus is present in the saliva for only a short time before onset of symptoms, symptoms of rabies should become unmistakable during this quarantine period, if the animal were infectious at the time of biting. Should the dog be killed before the development of typical infection the diagnosis may be questionable, whereas in the absence of symptoms the animal may be released after the quarantine period. Should the suspect animal not be apprehended or the diagnosis in the animal be uncertain, vaccination is mandatory following dog bite. Since vaccination against rabies is not without danger, the establishment of real human risk is highly desirable.

**Vaccination.** The Semple rabies vaccine, which consists of a phenolized emulsion of brain containing fixed virus, is the most widely used preparation. It is given in daily doses for fourteen days. Vaccines may, however, be inactivated by ultraviolet light, chloroform and other procedures. Pasteur originally used a vaccine consisting of a suspension of dried spinal cord of infected rabbits, which contained living fixed virus. The dosage was increased by using cords dried for progressively shorter periods of time. Vaccination against rabies is an effective preventive measure against the disease and should be used in man following exposure and in dogs as a prophylactic procedure. Postvaccinal encephalitis and paralysis, however, are a sufficient hazard to limit use of the vaccine in man to cases of real exposure.

**Control.** The control of rabies is dependent upon its diagnosis and elimination in animals. Elimination of stray, exposed and diseased animals and licensing, vaccination and quarantine of dogs are effective. Cooperation in the matter of identification, apprehension and quarantine of animals that have bitten human beings is extremely important.

**Poliomyelitis.** Poliomyelitis (infantile paralysis) is an acute viral infection of man characterized in typical cases by the development of muscular paralysis of varying degrees of severity. The clinical features have been recognized since the descriptions by Heine (1840) and Medin (1891), but the infectious nature of poliomyelitis was not established until 1908–1909 when Landsteiner, Popper and Flexner and Lewis transmitted the disease to monkeys and established its viral etiology.

Poliomyelitis is an acute febrile disease which begins after an incubation period estimated to be approximately two weeks, with preparalytic symptoms of fever, malaise, prostration, muscular pains, headache, often vomiting and signs of involvement of the central nervous system. Within a few days more or less extensive muscular paralysis appears. The paralysis affects primarily muscles of the limbs and trunk innervated by the spinal nerves (anterior spinal poliomyelitis) but may involve muscles supplied by nerve cells located in the brain stem (bulbar poliomyelitis). The lesions of the brain and spinal cord consist of foci of inflammation about the blood vessels and injury and destruction of the nerve cells. The neurons most susceptible to injury are the large motor nerve cells of the anterior horns of the spinal cord and brain stem (hence anterior and bulbar poliomyelitis) giving rise to flaccid paralysis. With recovery from the acute disease the injured neurons recover function and there is improvement in the paralysis, whereas the loss of completely destroyed cells is permanent and results in permanent paralysis. While the typical case is characterized by development of paralysis, the frequent occurrence of the mild, nonparalytic type of infection has been recognized in recent years. The disease has been noted to affect otherwise healthy, well-nourished individuals and in some instances follows tonsillectomy.

**The Virus.** The virus of poliomyelitis (*Legio debilitans*) has recently been classified in a single genus with the agent of human lymphocytic choriomeningitis (*L. erebea*) and Theiler's mouse poliomyelitis (*L. muris*). The virus is small in size, with an estimated diameter of 8–12 m $\mu$ , and appears to be protein in nature or at least is closely associated with the lipoproteins of infected tissues. The agent is inactivated by heat, ultraviolet light and chemical oxidants, such as chlorine, but is resistant to glycerol and ether.

Poliomyelitis naturally affects only man, but it may be transmitted experimentally to monkeys by intranasal and intracerebral inoculation. Recently certain strains of virus (particularly the Lansing strain) have been transferred to the cotton rat and to mice, thus providing an important method for laboratory study. In man the virus is regularly demonstrable in the nervous tissue of patients dying of the disease and may be found in the upper respiratory passages.



lymph nodes, tonsils, and in the feces. Experimental studies in monkeys indicate that the virus may enter the body through the mucosa of the nasopharynx or the intestinal tract and that it spreads from these areas to the spinal cord, brain and brain stem along the nerve fibers of the peripheral nerves as well as those of the central nervous system. The virus appears to spread particularly along the nonmyelinated autonomic fibers.

**Epidemiology.** The actual mode of transmission of poliomyelitis is not understood. As stated above the virus appears to enter through the nasopharynx or intestinal tract and it has been isolated from these locations as well as from nasopharyngeal washings and feces of patients and healthy contacts. It has also been demonstrated in sewage during an epidemic of the disease. However, water transmission has not been proved and it is not indicated by the distribution of cases of the disease (see Chapter 45). Milk has occasionally been implicated, but it appears to be of minor importance. The role of insects, particularly flies, and of contaminated food is uncertain, although virus has been demonstrated from both sources during an epidemic. The high seasonal incidence in late summer and autumn is, furthermore, compatible with insect transmission. However, the bulk of evidence from epidemiological studies indicates transmission by contact with patients or by healthy carriers of virus. The wide distribution and relatively low infectivity of poliomyelitis virus are indicated by the presence of sporadic cases, the low contact case rate, the high incidence of infection in children and the presence of neutralizing antibodies in the serum of adults who have not had the clinical disease. Although the disease is primarily a disease of children, adults are not infrequently infected. While the annual number of cases of poliomyelitis is only a fraction that of the more common communicable diseases, the infection is highly important because of its crippling effect and high fatality rate (5-25 per cent).

**Control.** Since the epidemiology is incompletely known, measures for control of poliomyelitis are directed against the several possible means of transmission. In general, isolation of recognized infections, avoidance of public meeting places, playgrounds, etc., good personal hygiene and insect control are practiced. During the poliomyelitis season tonsillectomy is best avoided and suspicious illness in children should be seen promptly by a physician. Sanitation of milk, food and water should not be neglected. Attempts to prevent infection in man by spraying the mucous membranes with solutions of antiseptics, alum and mixtures of tannic acid or picric acid and alum have been abandoned as unsuccessful.

**Immunity.** Using the neutralization test, antibodies against poliomyelitis virus may be demonstrated in a high proportion of serums from convalescent patients, contacts of patients and normal adults. Similar results are obtained with convalescent experimental animals. These antibodies are considered by most investigators to result from specific infection with the virus, although the view has been held that maturation immunity and nonspecific factors are responsible. Experimental animals may be protected from infection by inoculation of mixtures of serum and virus suspension. Immunity in poliomyelitis is, however,

peculiar in that antibody circulating in the blood may not provide protection and is not universally demonstrable following an attack of the disease. Furthermore, there appears to be some antigenic difference between strains of virus that convalescent serum is of questionable value in the prevention and treatment of poliomyelitis. The reasons for these discrepancies in the immune reaction in poliomyelitis are not entirely clear. It appears that the failure of some individuals to develop antibodies during the disease and of the serum to provide adequate protection may be related to the location of the virus within the cells of the nervous tissues and a failure of contact with the antibody or site of antibody formation.

Vaccination against poliomyelitis has in the past been unsuccessful and in human beings, actually dangerous since several cases of poliomyelitis occurred among vaccinated children. In animals neutralizing antibodies could be induced by vaccination, but protection against the disease was questionable. The vaccine consisted of virus suspension treated with either formalin or sodium ricinoleate. Indications are that newer methods of concentrating the virus and of inactivating by formalin or ultraviolet light perhaps may be successful, at least in experimental animals. There is no method for human vaccination at the present time.

**Lymphocytic Choriomeningitis.** Lymphocytic choriomeningitis is an acute, nonfatal, benign viral infection of the meninges and chorioid plexuses of the brain. The disease begins with symptoms which resemble influenza followed by headache, fever, stiffness of the neck, vomiting, stupor and other signs of meningitis. The spinal fluid contains many lymphocytes but no bacteria, and mild infections which do not exhibit evidence of nervous system involvement may occur. The virus appears to have a wide geographic distribution.

The virus of lymphocytic choriomeningitis (*Legio erebea*) may be demonstrated in the blood and spinal fluid during the early stages of the disease following inoculation of animals. *Legio erebea* is pathogenic for monkeys, mice and guinea pigs and has been found as a natural infection in animals. The gray house mouse develops a mild or latent infection and appears to be a natural reservoir in the infection has been reported in mice trapped in buildings in which human cases had occurred. Furthermore, a high proportion of domestic mice appear to be naturally immune to experimental infection. In experimentally inoculated animals the virus is found generally distributed in the body and is present in the blood, urine and respiratory secretions. Mice and monkeys develop signs of infection of the nervous system, whereas guinea pigs exhibit generalized debility usually without nervous symptoms, although histological examination reveals some involvement of the meninges.

Immunity is associated with the presence of neutralizing and complement-fixing antibodies. The complement-fixation reaction is particularly interesting in that a soluble antigen which is active in the test has been demonstrated in association with the virus.

Transmission of lymphocytic choriomeningitis by *Aedes aegypti* mosquito and the wood tick *Dermacentor andersoni* has been shown experimentally, and



virus has been found to survive throughout the life cycle in the latter rodent. Body lice and bed bugs are also possible vectors, and the *in utero* transmission of virus from infected female mice to their offspring has been recorded. Mice may also be infected by contact with diseased animals since the virus is present in feces, urine, semen, saliva and nasal secretions.

**The Viral Encephalitides.** The encephalitides are inflammatory and at times degenerative diseases of the brain substance. The symptoms of encephalitis are general ones of nervous system involvement (headache, fever, stiffness of neck, tremors, disorientation, stupor and coma) or of injury in specific regions within the nervous tissue. The disease has received the common name sleeping sickness because of the prolonged stupor and coma. Recovery from viral encephalitis may be complete, although permanent damage may be produced in some instances and the mortality rate is generally high (10 to 50 per cent). The lesions of encephalitis are referable to edema, inflammation and damage of the nervous tissue and to primary injury of the small blood vessels of the brain. Increased numbers of lymphocytic or round cells are found about the blood vessels and scattered within the brain substance, and there are hemorrhages and changes in the nerve cells themselves. In postinfectious encephalitis there is loss of the myelin sheath about the nerve fibers. Unlike the viruses of poliomyelitis and poliomyelitis, which directly injure the neuron and are primarily neurotropic, the encephalitis viruses are less neurotropic in that pathology is produced in both the nervous and vascular tissues. Furthermore, these viruses may be generally distributed in the body outside of the nervous system.

The infectious encephalitides are commonly classified into three groups: Type A is von Economo's disease or encephalitis lethargica; Type B is composed of the epidemic encephalitides of known viral etiology, such as St. Louis encephalitis, equine encephalomyelitis and Japanese B encephalitis; the third group includes those of other etiology, such as may be caused by herpes, rubella, measles and rubeola. In addition, there are the postinfectious and postvaccinal encephalitides, protozoal and other infections, as well as those caused by chemicals, such as lead and mercury, and some of completely obscure etiology. The postinfectious encephalitis following virus diseases, such as measles, mumps, chickenpox and influenza, may be the result of invasion of the central nervous system, although in these and postvaccinal encephalitis there is injury to the myelin sheath of the nerve fibers rather than inflammation.

**Von Economo's Lethargic Encephalitis.** Lethargic encephalitis has been recognized as an epidemic as well as a sporadic disease, a prominent acute symptom of which is lethargy. Although the disease had been recognized previously, von Economo carefully described and named it during an epidemic in 1916. Lethargic encephalitis is recognized in many countries including the United States, and it is generally sporadic with the major incidence during the warmer months of the year. The symptomatology results from the location of lesions in many different areas of the brain. Postencephalitic parkinsonian symptoms with tremors and gait disturbances are prominent among the late

effects of the disease. The lesions in the brain are both inflammatory and destructive.

The etiology and epidemiology of this type of encephalitis are obscure although the disease appears to be infectious and probably of viral etiology.

**St. Louis Encephalitis.** In the summers of 1932 and 1933 outbreaks of encephalitis occurred in central Illinois and St. Louis, Missouri. In St. Louis the new disease was first recognized in the periphery of the city and later involved the central metropolitan area. Since its description St. Louis encephalitis has been recognized in the western United States and serum neutralization tests indicate a wide distribution of the virus. The symptoms of encephalitis appear suddenly or after a prodromal period of malaise.

The virus of St. Louis encephalitis is infectious for monkeys, hamsters and mice and may be cultivated in the chick embryo and in tissue culture. Virus may be demonstrated in the blood of domestic fowl following experimental inoculation but these animals remain symptom-free. The virus is immunologically related to but not identical with the virus of Japanese B encephalitis and West Nile virus and, although immunologically distinct, exhibits interference with encephalomyelitis virus in the chick embryo. Neutralizing or protective antibodies appear in human and animal serums following infection and together with the isolation of virus have indicated the epidemiology of the disease. Thus, antibodies have been demonstrated in the blood of horses and domestic fowl in endemic areas and virus has been isolated from *Culex* mosquitoes (*C. tarsalis*) and chicken mites (*Dermanyssus gallinae*) in nature. Several species of mosquitoes, chicken mites and ticks (*Dermacentor variabilis*) have been shown experimentally to transmit the virus. Furthermore, the chicken mite and tick transmit the virus through the egg from one to the next generation. As a result of such investigations St. Louis encephalitis virus appears to be maintained in nature in the domestic chicken and chicken mite and to be transferred from one to another fowl by the mite and mosquitoes. Humans and other mammals appear to be infected by the bite of infected mosquitoes.

Vaccines capable of immunizing animals and presumably man may be produced by inactivating suspensions of virus by formalin and ultraviolet irradiation. Extensive trials of human vaccination have, however, not been made.

**Japanese B Encephalitis.** Epidemic Japanese summer or B encephalitis is clinically, pathologically and epidemiologically similar to St. Louis encephalitis. Furthermore, the viruses of these diseases and West Nile encephalitis of Africa are all immunologically related. The disease has been recognized as a highly fatal (approximately 60 per cent) epidemic infection in Japan for over seventy years and has been reported from other islands and the continent of Asia. Culicid mosquitoes appear to transmit the disease and domestic fowl and mammals may be infected.

**Equine Encephalomyelitis.** Equine encephalomyelitis was first described as an epidemic disease among horses in the United States in 1930. The disease is widespread among animals in the United States and elsewhere in North



Central and South America, Europe and Japan, and is an important cause of human encephalitis. Encephalomyelitis has great economic as well as public health importance.

Three distinct types of equine encephalomyelitis viruses are recognized; the eastern type is a highly virulent virus found in the eastern United States, the western type is less virulent and more widely distributed and the Venezuelan type is found in South America and elsewhere. Cross-neutralization tests indicate a lack of protection between these several types of virus. Encephalomyelitis virus has an approximate diameter of 40 to 50  $m\mu$  and appears to be nearly spherical in shape. Chemical studies have revealed a lipoid-nucleoprotein complex and the virus is unusual in its ribose-type nucleic acid and high lipid content. The viruses of encephalomyelitis are infectious for man and a wide variety of laboratory, domestic and wild mammals and birds and may be cultivated in the chick embryo. Both experimental and natural infections of culicine mosquitoes (*Culex* and *Aedes*) have been demonstrated with Western virus, and natural infection of bugs of the genus *Triatoma* and of the mites of domestic and wild birds has been reported. It thus appears that equine encephalomyelitis virus is epidemiologically similar to St. Louis virus, is arthropod-borne, chiefly by mosquitoes, and infects man and equines accidentally. The most important animal reservoir of infection would appear to be domestic and wild birds.

All three types of encephalomyelitis virus are infectious for man, producing a disease which resembles St. Louis encephalitis. Human infection occurs epidemically and sporadically in the summer months and appears concomitantly with the disease in animals. Subclinical human infection appears to be infrequent. Neutralizing and complement-fixing antibodies may be demonstrated in convalescent serum.

Vaccination of horses and mules against encephalomyelitis has received extensive application in the United States and appears to reduce significantly the incidence of disease. Formalinized suspensions of chick embryo virus may also be used for protection of human beings exposed to unusual risk of infection, e.g., laboratory personnel. Hyperimmune rabbit serum has been found to increase the survival rate of mice and guinea pigs experimentally infected with Western virus, and equine serum has been used with inconclusive results in treatment of human disease.

**Russian Spring-Summer Encephalitis.** Russian encephalitis occurs in forested areas of the eastern provinces of the Soviet Union during the spring and summer months. Man, rodents and ticks of the genus *Ixodes* are infected. The virus appears to be maintained within the tick and is transmitted from tick to the next generation through the egg, although widespread infection of forest animals is indicated by neutralizing antibodies in the blood serum. The virus is immunologically related to that of louping ill disease of Scotland.

**Louping Ill.** Louping ill is a tick-borne (*Ixodes ricinus*) viral encephalitis of sheep in Scotland and adjacent counties of England. The disease is characterized by an ataxic and stumbling gait resulting from encephalomyelitis of

the cerebellum with necrosis of the neurons in this area. Man, cattle and horses are occasionally infected and mice and monkeys may be infected experimentally.

**Other Types of Encephalitis.** A virus was isolated from the Australian encephalitis known as X disease, which occurred following World War I. This virus has not been maintained, hence any relationship to other encephalitic viruses is conjectural. The B virus was isolated from a laboratory worker who became infected following a monkey bite but it has not otherwise been reported to cause human illness. West Nile virus is a cause of human encephalitis in Africa. The virus is antigenically related to the viruses of St. Louis and Japanese encephalitis.

In addition to the viral encephalitides which may affect man, lower animals are subject to similar infections, such as the encephalitis of foxes and Bordeaux disease. The latter is a viral encephalitis of horses in Europe which in its symptomatology resembles equine encephalomyelitis, although the virus appears to be a separate agent.

### THE PSITTACOSIS-LYMPHOGRANULOMA VENEREUM GROUP

Viruses similar in morphology, developmental cycle and immunology to those causing psittacosis and lymphogranuloma venereum recently have been classified with the rickettsiae, the bartonellas and the agents of trachoma and inclusion conjunctivitis in the Order Rickettsiales. The genus *Miyagawanella* includes the viruses of lymphogranuloma venereum (*M. lymphogranulomatis*), psittacosis (*M. psittacii*), ornithosis (*M. ornithosis*), human pneumonitis (*M. pneumoniae*), *M. louisianae*, *M. illinii*) and pneumonitis of lower animals (*M. bronchopneumoniae* of mice and *M. felis* of cats). Although both psittacosis and lymphogranuloma venereum had been previously described, the nature of the causative agents was first recognized in 1930. The other viruses and the relationships among these disease agents have been recognized more recently.

These viruses appear to multiply chiefly but not exclusively within living cells, and in their general properties appear to be intermediate between the rickettsiae and the more typical viruses. In morphology they are essentially similar. Coccoid elementary bodies, which appear to represent the virus particles, may be observed in smears of infected material stained by Giemsa, rickettsial stains or Machiavello's basic fuchsin-methylene-blue methods. These viruses undergo a cyclical development within the cytoplasm of infected cells, or upon the cell surface in some instances, in which the elementary body enlarges rapidly to the initial body stage, and then a vesicle or plaque consisting of large numbers of elementary particles embedded in a clear matrix is formed. Rupture of the vesicle liberates infectious particles. The viruses are infectious for a variety of mammals and birds and may be cultivated in the chick embryo and in many instances in tissue culture. A specific toxic fraction which appears to be antigenic has been described for the psittacosis, lymphogranuloma, ornithosis and mouse



pneumonitis viruses in that infected chick embryo yolk sacs are highly toxic to man. The viruses of the group are unique in their susceptibility to one or both of the sulfonamide drugs and penicillin. Streptomycin is ineffective. Aureomycin is ineffective.

These viruses appear to possess at least one antigen in common and cross reactions are obtained in the complement-fixation reaction. A group-reacting antigen of *M. lymphogranulomatis* has been separated and appears to be ether-soluble and possibly a glucolipid. Neutralizing antibodies have been difficult to demonstrate. However, immune serum from chickens has been found to possess specific neutralizing antibodies. By this method the separate identity of most of the group may be demonstrated, although the test indicates the close relationship of ornithosis virus with one isolated from human disease (meningopneumonitis virus). By means of the Frei hypersensitive skin test (see below) human pneumonitis virus appears antigenically related to *M. lymphogranulomatis*.

**Psittacosis and Ornithosis.** Psittacosis and ornithosis are enzootic (endemic) infections of birds, which are transmissible to man. Formerly regarded as a rare human disease acquired from sick parrots, since 1930 the infection has been found among birds in many geographic areas and as an epidemic or sporadic disease of man. In comparison with the human communicable diseases, ornithosis is, however, infrequently recognized. Psittacosis is a disease of wild and domestic psittacine birds (parrots, parakeets and related birds), whereas ornithosis affects other domestic and wild species (pigeons, petrels and others). The diseases appear to be essentially similar in their epidemiological and clinical characteristics, but are caused by distinct although related viruses.

Psittacine birds appear to become infected as fledglings in the nest from healthy carriers, or they may acquire the disease at a later age. Transmission and development of symptoms is favored by factors such as crowding, unhygienic conditions, shipping and exposure to cold. Newly infected or, under adverse conditions, latently infected birds may develop active disease characterized by depression, ruffling of the feathers, weakness and discharge from the nose and ocular vent. The liver has many foci of necrosis and the spleen is characteristically enlarged. The disease may be highly fatal.

The disease is readily communicable and human beings are highly susceptible to psittacosis. Human infection appears to occur through the respiratory tract by inhalation of virus in dust particles or droplets from acutely ill patients. However, the disease has not become established in the human population and man-to-man transmission is unusual. Psittacosis is an acute pneumonitis with a mortality between 20 and 30 per cent of cases. The lungs show an atypical lobular pneumonia with patchy areas of consolidation. Histologically there is predominance of the large mononuclear phagocytic cells with edema and an early infiltration of fibrin and polymorphonuclear cells. In addition to the lung lesions there may be general enlargement of the lymph nodes and spleen, inflammation and necrosis of the liver, degeneration of the heart muscle and small hemorrhages.

of the brain and other tissues. Human infection has occurred in Europe, North and South America, Australia and elsewhere.

The virus of psittacosis is present in the tissues and often in the discharge of infected birds and is found in the lung lesions, the sputum and the blood of patients. The coccoid elementary bodies (L.C.L. or Levinthal-Cole-Lillie bodies) are seen in impression smears of infected tissues and appear to represent virus particles. Infection may be produced by intranasal, intracerebral, intravenous, or intraperitoneal inoculation of mice and the virus may be cultivated in tissue culture and in the chick embryo.

Immunity is evidenced by the development of complement-fixing antibodies in the serum during both human and animal infections and may be produced artificially by vaccination with virus suspensions by the intramuscular route.

The diagnosis of psittacosis and ornithosis depends upon the isolation of the causative virus and the demonstration of an immune reaction to the virus. In human infections with this group a rise in titer of complement-fixing antibodies in the serum may be observed, which together with the typical history and clinical findings are diagnostic. Because of the high infectivity of these viruses isolation and study of them is inadvisable except in properly equipped virology laboratories.

Psittacosis and ornithosis viruses are susceptible to high concentrations of penicillin and some strains of the former may be inhibited by sulfonamide drugs. Control measures against the natural infection in wild birds are largely ineffective. However, regulation of importation and sale and the domestic breeding of birds help to protect the human population.

**Pneumonitis.** Pneumonitis, indistinguishable from other forms of atypical pneumonia (Chapter 27), is produced in man by several members of this group. Thus, human psittacosis and ornithosis primarily affect the lungs. In addition several other viruses have been isolated from epidemic or sporadic human pneumonias, and similar viruses have been isolated from naturally infected mice and cats. Transmission of the animal viruses appears to be by contact.

**Lymphogranuloma (Lymphopathia) Venereum (Lymphogranuloma Inguinale).** Lymphogranuloma venereum is a human venereal disease (Chapter 44) which has a wide geographic distribution but is more frequent in tropical and subtropical climates and in the Negro race than in temperate zones and the Caucasian race. The initial lesion of the genitalia is trivial and common and escapes notice. However, within a few days to a month infection of the regional lymph nodes is noticed, with adenitis, often with pus formation, in the inguinal lymph nodes of the male and the pelvic and perirectal nodes in the female. The disease is a systemic infection which may give rise to chronic granulomatous inflammatory lesions in many locations. Thus, inflammatory tumors with purulent sinus tracts may occur about the female genitalia (esthiomene), and lymphogranulomatous colitis with ulceration and stricture of the rectum are not infrequent. Meningitis, conjunctivitis and other extragenital lesions may be observed. The virus is present in the urogenital and rectal lesions, the lymph



s and exudates therefrom. It has also been demonstrated in meningitis conjunctivitis.

Patients and infected animals develop complement-fixing antibodies, and hypersensitivity may be demonstrated in the Frei test. Originally the Frei test is performed by the intradermal inoculation of a heat-inactivated suspension as from an infected lymph node. At the present time, however, commercial antigen (Lygranum) prepared from infected yolk sacs of chick embryos is available. A positive reaction consists of the delayed appearance of redness and induration of the skin at the site of inoculation and in the presence of symptoms of diagnostic significance. The virus of lymphogranuloma venereum is susceptible to the sulfonamide drugs and to aureomycin.

## THE PATHOGENIC HIGHER FUNGI

The relationship of fungi to disease of man, animals and plants was recognized before demonstration of the pathogenicity of bacteria. Thus, the causative agents of thrush (*Candida albicans*), several of the ringworm fungi and favus (*Achorion schoenleini*) were identified during the early nineteenth century. However, the importance of the bacterial and viral infections as causes of serious illness and death is far greater than that of the fungus diseases, the frequent type of which, such as ringworm, are not a menace to life. Consequently, in comparison with the bacteria and viruses the pathogenic fungi received little attention until recent years. Modern medical mycology may be said to have begun with the work of Sabouraud during the early part of the twentieth century. With present control of many of the serious communicable diseases and the better identification of fungi, recognition of the mycoses and, hence, their importance in human disease are increasingly important.

**Classification of Fungus Diseases.** Infections with fungi are named **mycoses**, usually with a prefix indicating the causative fungus; thus **actinomycosis** is infection with *Actinomyces*, and **blastomycosis** and **coccidioidomycosis** are caused by *Blastomyces* and *Coccidioides*, respectively. At times the disease name is somewhat abbreviated, as in **aspergillosis**, **histoplasmosis** and **sporotrichosis**, which refer to infection with *Aspergillus*, *Histoplasma* and *Sporotrichum*. In other instances the disease name refers to the location of infection within the body, such as **dermatomycosis** (infection of the skin) and **onychomycosis** (infection of the nails).

With few exceptions the fungi pathogenic for man and animals belong to the *Hyphomycetes* (see Chapter 5). Further subdivision is conveniently made on a clinical basis and the individual species are recognized by cellular and color morphology. Fungus infections are divided into two major groups, the **superficial mycoses**, which are relatively benign infections of the skin, nails, hair and mucous membranes, and the **systemic mycoses**, which may injure many tissues of the body, including the skin, lungs, bones, and central nervous system. The superficial mycoses, such as the common dermatomycoses, are frequent infections, whereas the more severe, often highly fatal systemic mycoses are relatively infrequent diseases. An outline of the more important fungus diseases is given in Table 18.

The pathogenic fungi are a heterogeneous group of microorganisms. The



include the actinomycetes, which are best regarded as transitional forms between bacteria and the higher fungi, yeasts and yeast-like organisms, such as *Candida*, mold-like fungi and molds, such as *Aspergillus*. The characteristics of specific organisms are described in the discussion of the specific diseases.

**Methods for Recognition and Study of Fungi.** The specific diagnosis of fungus diseases is made by the demonstration and identification of the causative fungi from infected tissues. The methods include direct examination of tissues and body fluids, cultivation of the fungi, animal inoculation and immunological methods.



Fig. 171. Branching hypha seen in potassium hydroxide preparation of the skin (Fig. 275). (From Smith and Martin: *Zinsser's Textbook of Bacteriology*, 9th ed., Lippincott-Century-Crofts, Inc.)

**Direct Examination.** The demonstration of fungi in sputum, pus, the tissues and scrapings from nails, skin and hair is extremely useful in the diagnosis of fungus infection. Usually, however, the fungi are not specifically identified by this method so that cultivation is required. Preparations of wet, unstained materials are valuable in examination of pus, skin scrapings and hair. It is usually necessary to mount materials in strong alkali (preferably 10 per cent sodium or potassium hydroxide) in order to dissolve the tissue elements and allow better visualization of the fungus. Several other mounting fluids, such as Amann's solution of phenol, lactic acid, glycerol and cotton blue in water, or suspension of the specimen in water may also be used.

Stained fixed smears of exudates are less diagnostic than in bacterial infections. However, *Candida albicans* and *Aspergillus* may be seen in smears stained by Gram's method, and *Histoplasma* may be found in Giemsa-stained smears of aspirated bone marrow. Sections of tissues may be stained by several methods, such as Gram-Weigert, Giemsa or hematoxylin and eosin.

Examination of infected hairs under ultraviolet light in the dark is useful in recognition of tinea or ringworm of the scalp. *Microsporum* and certain other fungi when observed by this method produce fluorescence of different colors. The

TABLE 18. OUTLINE OF FUNGUS INFECTIONS

DISEASE GROUP	DISEASES	CAUSATIVE FUNGI	GENERAL DESCRIPTION
Superficial mycoses of skin, nails, hair (dermatomycoses) and mucous membranes	Tinea or ringworm	<i>Microsporum spp.</i> <i>Trichophyton spp.</i> <i>Epidermophyton spp.</i>	Tinea may involve any area of the nails, the hair follicles. <i>Tinea capitis</i> : ringworm of scalp, ally occurs in children. <i>Tinea barbae</i> : barber's itch (sycosis bearded area. <i>Tinea corporis</i> : ringworm of smooth glabrous skin. <i>Tinea pedis</i> : athlete's foot. <i>Tinea cruris</i> : Dhobie itch; ringworm of groin, axilla, breasts. Onychomycosis: tinea of nails.
	Favus	<i>Achorion (trichophyton) schoenleini</i>	Favus of scalp, occasionally of other skin. The disease is a form of ringworm with crust (scutula) formation.
	Chromoblastomycosis (dermatitis verrucosa)	<i>Hormodendrum spp.</i> <i>Phialophora sp.</i>	Wart-like fungus infection of skin, usually of lower leg.
	Rhinosporidiosis	<i>Rhinosporidium seeberi</i>	Disease of cattle and horses transmissible to man. Infection of the skin and mucous membranes, particularly of nose, with formation of inflammatory growths or polyps containing the fungus.
	Moniliasis (thrush, onychomycosis, cutaneous moniliasis, monilial vaginitis, systemic moniliasis)	<i>Candida (Monilia)</i>	Localized cutaneous moniliasis, monilial intertrigo of axillae, groin, feet, onychomycosis and paronychia of nails. Perlèche of angles of the mouth. Erosio interdigitale of interdigital spaces of the hands. Oral thrush: infection of mouth of infants. Vaginitis. Generalized and systemic moniliasis (uncommon). Generalized cutaneous moniliasis. Systemic moniliasis: lungs, meninges.
Systemic mycoses	Aspergillosis	<i>Aspergillus fumigatus</i>	Superficial infection of external ear. Pulmonary aspergillosis simulates tuberculosis.



TABLE 18. OUTLINE OF FUNGUS INFECTIONS (Concluded)

CLASS GROUP	DISEASES	CAUSATIVE FUNGI	GENERAL DESCRIPTION
Fungal diseases	Sporotrichosis	<i>Sporotrichum schencki</i>	Usually cutaneous abscess and ulcer with infectious nodules in regional lymphatics; uncommonly generalized subcutaneous form.
	Actinomycosis	<i>Actinomyces bovis</i> <i>Nocardia asteroides</i>	Lumpy jaw of cattle; superficial and systemic actinomycosis of man. Chronic granulomatous, spreading infection which may involve any tissue of the body. Infection of face and neck, chest and abdomen are most frequent.
	Mycetoma (Madura foot)	<i>Actinomyces spp.</i> , Fungi imperfecti ( <i>Monosporium</i> and <i>Madurella</i> ). Possibly other fungi.	Chronic ulceration and deep spreading inflammation and abscesses of feet. Onset often follows injury. Occurs chiefly in India (Madura).
	<i>Erysipelothrix</i> infection (erysipeloid)	<i>Erysipelothrix rhusiopathiae</i>	Swine erysipelas; human infection (erysipeloid) is usually a deep localized mycosis acquired from infected animals.
	Rat-bite fever, Haverhill fever	<i>Actinomyces muris</i> ( <i>Streptothrix</i> ; <i>Streptobacillus moniliformis</i> )	Normal parasite of rats and mice; one form of rat-bite fever in man.
	Blastomycosis	<i>Blastomyces dermatitidis</i>	Cutaneous and systemic (pulmonary) infection. Chronic, progressive, granulomatous nodules, abscesses and ulcers.
	Torulosis (cryptococcosis; European blastomycosis)	<i>Torula histolytica</i> ( <i>Cryptococcus neoformans</i> )	Abscesses of skin; infection of lungs; <i>Torula meningitis</i> .
	Coccidioidomycosis (coccidioidal granuloma, valley fever, desert rheumatism)	<i>Coccidioides immitis</i>	Primary pulmonary infection resembling tuberculosis; generalized progressive coccidioidomycosis; cutaneous infection.
	Histoplasmosis	<i>Histoplasma capsulatum</i>	Generalized infection of reticulo-endothelial system, with lesions in many other tissues. Infection often occurs in the lungs, deep tissues, bones and skin.

method is of particular value in detecting infected hairs before the lesion is grossly visible and in the study of isolated cultures.

**Cultivation.** The nutrition and cultivation of fungi have been discussed in Chapter 5. In studying pathogenic fungi it is important that cultural media permit isolation from mixtures with bacteria and that the medium be complete in composition so that colonial morphology and pigmentation are reproduced. The medium most commonly used in medical mycology is Sabouraud's 2 per cent glucose agar, pH 5.6. This medium is particularly valuable in isolation and study of the dermatophytes and will support the growth of most fungi, but it is not satisfactory for some exacting species. Many fungi require a prolonged incubation period of many days to several weeks for growth. Material from lesions can usually be cultured directly on slants or plates of Sabouraud's agar, although preliminary treatment of hair and skin with 70 per cent alcohol may reduce the number of contaminating bacteria. The study of single giant colonies (4 weeks old) is of value in the identification of fungi, as is the observation of pure cultures, grown in a narrow layer between a microscopic slide and cover glass. Fungi grow luxuriantly on blood agar, and this medium is recommended for the cultivation of *Histoplasma capsulatum* and other exacting organisms. The colonial and cellular morphology of the fungus should be determined by gross and microscopic examination.

**Cultural Reactions.** Cultural reactions, particularly pigment production, fermentation of carbohydrates, may be diagnostic of certain fungi. Thus, many molds and yeast-like fungi produce characteristically pigmented colonies. Utilization of carbohydrates has been suggested as a basis for classification of the ringworm fungi and for the yeast-like members of the genus *Candida*.

Many of the causative agents of the deep mycoses are pathogenic for laboratory animals. Inoculation of animals may thus be helpful in the isolation and identification of these organisms.

**Pathogenicity.** The mechanisms by which fungi produce disease are not clearly understood. Toxin production has received considerable attention, although exotoxins such as those produced by bacteria have not been demonstrated. The toxicity for animals of the cell substance of culture filtrates of some molds, actinomycetes and higher fungi is well known. Thus, antibiotic substances produced by some fungi may be highly toxic for the animal body, and among the higher fungi certain species of mushrooms, particularly of the genus *Amanita*, produce deadly toxins (see Chapter 44). The endotoxic substance of *Aspergillus fumigatus* produces hemorrhages, edema and damage of the kidneys and liver in rabbits and guinea pigs. In general, however, fungus infections are not markedly toxic, but are characterized by invasion and destruction of tissue and by inflammation.

The dermatophytes possess relatively little invasive power and remain localized in the skin. In the systemic mycoses, on the other hand, these may be spread from the original site of invasion to many parts of the body. The diseases



ally chronic with formation of granulomata and abscesses similar to those of tuberculosis and syphilis.

**Immunity.** Immunity in the fungus diseases is seldom sufficient to result in spontaneous cure of the infection. On the other hand, fungus diseases are often rapidly fatal and many are limited or superficial in nature. This chronicity and lack of tissue invasion may be due in part to host resistance as well as to the limited invasive power of the microorganism. In addition, certain fungi of the dermatophyte group are remarkably limited in their host range, and *Microsporum audouinii*, a cause of ringworm of the scalp in children, does not attack adults. Furthermore, ringworm of the scalp generally heals spontaneously at maturity, probably as a result of the presence of lipoidal substances in the adult scalp which are fungicidal for *M. audouinii*.

Immune bodies against fungi are in general produced in low titer and are demonstrated with difficulty. Studies of the agglutination, precipitation and complement-fixation reactions using the serums of patients and immunized animals have, however, been of value in some fungus infections. The immune response appears to be greater in the systemic mycoses than in the superficial infections. Thus, in sporotrichosis the complement-fixation and agglutination reactions may be positive; the results of the complement-fixation reaction may be significant in systemic coccidioidal infection, histoplasmosis and blastomycosis, and agglutinins and precipitins may be produced against *Candida*. Immune serum is uniformly successful in fungus diseases.

Hypersensitivity to fungi and culture extracts is, on the other hand, frequently present, and allergic skin reactions are a diagnostic aid in many infections. Hypersensitivity is, therefore, of great interest to the medical mycologist. Hypersensitive skin tests using extracts of fungus cultures have been developed in ringworm (**trichophytin test**), blastomycosis (**blastomycin test**), coccidioidomycosis (**coccidioidin test**), histoplasmosis (**histoplasmin test**) and moniliasis (**oidiomycin test**), although positive reactions to coccidioidin occur so frequently in uninfected as well as infected persons that the test is of little or no value. The skin tests are performed by injecting the fungus extract into the skin; in a positive reaction, indicating hypersensitivity, an area of redness and induration appears at the site of the injection. The tests are thus analogous to the tuberculin reaction and they alone should not be taken as evidence of active infection.

In certain diseases there are manifestations of hypersensitivity other than skin reactions to injection of extracts of fungi. In the dermatomycoses spontaneous generalized skin eruptions, known as **dermatophytid** or **trichophytid**, occur during the course of the disease. These skin eruptions are generally considered to represent allergy, *i.e.*, hypersensitivity, of the skin and are generally associated with sensitivity to trichophytin. In animals experimentally infected with dermatophytes an increased inflammatory reaction may be observed following a second inoculation of fungus. This reaction may be looked upon as an immune or hypersensitive response. In addition, the association of some cases of

asthma and hay fever with hypersensitivity to fungus spores and air-borne has been reported. The responsible fungi include saprophytic molds and yeasts such as *Aspergillus*, *Penicillium* and *Alternaria*. In other instances rusts and smuts have been suggested as allergens in seasonal hay fever.

### TINEA AND DERMATOPHYTOSIS

#### *The Ringworm Group of Fungus Diseases*

The tinea or ringworm group of superficial mycoses is the most frequent of fungus infection. The group, which includes such well known infections as tinea capitis (ringworm of the scalp), favus, tinea barbae (ringworm of the beard), tinea glabrosa or corporis of the smooth skin and dermatophytosis of the feet, hands and nails, is characterized by infection of the keratinized portions of the skin (the superficial layers of epidermis, the hair and nails). Although systemic reactions, such as dermatophytid, may be present, and spread from one to another area of infection of the skin occurs, invasion of the deep tissues with granuloma and abscess formation is not characteristic of these diseases. Inflammation about the site of infection of the skin is usually mild with scaling and crust formation, although there may be considerable inflammation with pus formation and secondary bacterial infection. Despite their superficial nature, ringworm infections may be exceedingly resistant to treatment. Sensitivity to trichophytin is frequently present in infections caused by *Trichophyton* and is variable in the other dermatophytoses. Fungi of the ringworm group are transmissible from person to person and at times assume epidemic proportions. Tinea capitis is not infrequently epidemic among school children in the absence of proper hygiene; tinea barbae may be transferred among barber shop patrons and dermatophytosis and tinea of the feet (athlete's foot) may be widespread among athletes and residents of institutions or dormitories, at times affecting as many as 50 to 90 per cent of the members of the group.

The dermatomycoses are classified clinically into several types (Table I). They are caused by a number of species of fungi, which are members of several genera, *Microsporum*, *Trichophyton*, *Epidermophyton* and *Achorion*, and which are distinguished by their conidia and other morphological structures.

**Microsporum.** The genus *Microsporum* includes those fungi of the ringworm group which produce small (3–4  $\mu$ ) oidia or "spores" by fragmentation of the mycelium. Three species are of medical importance: *M. audouinii*, *M. canis* (*M. lanosum*) and *M. gypseum*. Microspora may be demonstrated by direct examination of the infected skin, hair follicles and hair shafts. In preparation of the skin, branching, segmented mycelium is observed; oidia are not seen in infected hairs, on the other hand, the mycelium is present within the hair shafts and oidia are observed in a mosaic pattern on the surface of the hair. In culture, microspora produce velvety or cottony mold-like colonies which vary in color from white to brown. Spindle-shaped fuseaux or macroconidia, which are the



and multicellular, are characteristic of the genus. Cultures also contain microconidia, pectinate hyphae and chlamydospores.\*

Microspora are important causes of ringworm of the scalp in children, although infection of the beard and glabrous skin also occurs. *Microsporum canis* is particularly frequent in Europe and is responsible for approximately half of the infections in the United States. Infection with *M. canis* is also prevalent in the United States, whereas *M. gypsum* is infrequent. It is epidemiologically important that *M. canis* and *M. gypsum* are natural parasites and pathogens of lower animals, particularly dogs and cats.

**Trichophyton.** Fungi of the *Trichophyton* group are the large (7-8  $\mu$ ) variety of dermatophytes. In direct preparations of skin these fungi appear as branched, segmented mycelium, which may be fragmented into arthrospores. Infected hairs *Trichophyton* spores occur in chains both within and on the surface of the hair. Smooth to powdery mold-like growth is produced in culture and may be white or shades of yellow, red, purple and brown. Microscopic examination of cultures reveals numerous microconidia in clusters on conidiophores arising singly along the hyphae, spiral hyphae, racquet hyphae and multiseptate macroconidia or fuseaux. Several species of *Trichophyton*, which differ in general characteristics, pigmentation and pathogenicity, are recognized, chief among which are *T. mentagrophytes* (*T. gypsum*), *T. rubrum* (*T. purpurcum*), *T. tonsurans* (*T. crateriforme*), *T. sabouraudi*, *T. rosaceum* and *T. violaceum*.

*Trichophyton* species are important causes of superficial mycosis of the glabrous skin (tinea glabrosa, tinea corporis, tinea circinata), the beard (barber's tinea barbae) and the scalp (tinea capitis), and are less often responsible for infection of the feet (tinea pedis), nails (onychomycosis) and groin (tinea cruris, jock itch, gym itch).

**Epidermophyton.** *Epidermophyton* does not invade the hair, but it is a frequent cause of mycosis of the skin and nails. In direct preparations only branch-segmented mycelium is seen. Cultures of *Epidermophyton* are greenish yellow in color and velvety in appearance, with radiating furrows. On microscopic examination, septate mycelium with chlamydospores and racquet mycelium are observed. The cultures produce only multiseptate macroconidia or fuseaux. There is only one species of *Epidermophyton*, *E. floccosum* (*E. inguinale*).

*Epidermophyton* is a frequent cause of "athlete's foot" or tinea pedis, tinea cruris and onychomycosis. It does not affect the glabrous skin, the scalp or the beard. Infection is usually superficial and limited in area unless secondary infection occurs. Sensitivity to trichophytin is usually absent.

**Achorion: Favus** (*Tinea favosa*). Favus is a form of ringworm of the scalp characterized by the formation of yellowish, cup-shaped crusts or scutula about infected hairs, which characteristically results in patchy baldness (alopecia) in the infected areas. Favus is most frequently observed in Europe and in the United States it is occasionally encountered among immigrants. In contrast to

The student is referred to textbooks of mycology for descriptions of the individual species *Microsporum*, *Trichophyton* and *Epidermophyton*.

inea capitis caused by *Microsporum*, spontaneous cure of favus does not occur at puberty.

The causative agent of favus, formerly known as *Achorion schoenleini*, is from the trichophyta only in the type of infection which it produces and is frequently classified as *Trichophyton schoenleini* on the basis of its morphological and cultural characteristics. Direct examination of the favic scutula and infected hairs reveals masses of mycelium within the scutulum and mycelium and large fungus spores within the hair. Cultures are initially waxy, yellowish and deeply furrowed, but later become velvety and mold-like in appearance. Microscopic examination reveals irregular mycelium and swelling of the hyphae, chlamydospores and the characteristic branched, tree-like mycelial structures known as favic chandeliers.

Treatment and control of the dermatomycoses may present difficult clinical and public health problems. Immunological methods are of no therapeutic value except that occasionally desensitization may be advisable. Treatment methods include use of suitable local ointments and solutions, chemical, mechanical or physical (x-ray) removal of infected nails, skin and hair and prevention of infection. In prevention of infection, swimming pool and shower sanitation, exclusion of infected persons from use of such facilities, examination of school children for infection, and good personal hygiene are important.

### CHROMOBLASTOMYCOSIS

Chromoblastomycosis is a chronic superficial mycosis characterized by warty lesions of the skin of the lower extremities with scarring and edema of deeper tissues. The disease is relatively rare, affects males more frequently than females and appears to have a wide geographic distribution, including the United States. It has been suggested that the causative fungi, *Hormodendrum pedunculatum*, *H. compactum* and *Phialophora verrucosa*, are naturally saprophytic or parasitic on wood and that infection is acquired from these sources. In potassium hydroxide mounts of material from the lesions the fungi are observed as brownish, thick-walled, yeast-like cells, which reproduce by fission, not by budding. Cultures grow slowly and produce deeply pigmented brown to olive black mold-like colonies after several weeks. There is septate mycelium, and conidia are produced on conidiophores, which may be straight, club-shaped or characteristically branched. The immunology is incompletely known, although complement-fixing antibodies have been demonstrated.

### RHINOSPORIDIOSIS

Rhinosporidiosis, caused by *Rhinosporidium seeberi*, is a superficial mycosis affecting particularly the mucous membranes of the nose and eyes and less often the skin and other mucous surfaces. Rhinosporidiosis occurs in a wide geographic distribution, including the southern and central United States. Infection occurs



ally in equine and bovine animals and in man, and is characterized by the development of polyps containing inflammatory tissue and the large, heavy-walled fungus cysts (sporangia) filled with small spores. The fungus has not been cultivated and the mode of transmission is obscure, although water transmission appears on epidemiological grounds to be a possibility.

### MONILIASIS

Moniliasis is an acute or subacute infection caused by *Candida* (*Monilia*), usually *Candida albicans*. Two forms of the disease are recognized, the frequent superficial mycosis of the mucous membranes, skin and nails and the relatively rare systemic infection. The fungus is world-wide in distribution, affects all age



Fig. 172. Moniliasis. Segmented mycelium and yeast-like cells are seen in a smear of vaginal moniliasis. The small rod-shaped organisms are Döderlein's rods. Gram's stain. (Magnification approximately  $\times 750$ .)

groups and both sexes and may be isolated from normal as well as from diseased organisms.

Infection of the skin most commonly affects the moist, intertriginous areas (axilla, groin, the inframammary area, the gluteal fold, the webs of the fingers and toes) and the corners of the mouth (perlèche). The lesions of monilial infection of the skin are painful or itching, reddened, exudative patches often with vesicles, pustules, scaling and crust formation. Infected nails become thickened, discolored and surrounded by an inflammatory reaction.

Moniliasis of the mucous membranes (**thrush**) is most frequently oral or vaginal, although in rare instances extensive lesions of other mucous surfaces are observed. Oral thrush is most common in nursing infants, and less frequently occurs in adults. Cream-colored surface patches of loosely adherent false membrane and exudate containing the fungus are characteristic of the infection. Fungus hyphae are found in abundance in direct preparations from infected mucous

membranes. Systemic moniliasis is relatively uncommon, although infection of the bronchi and lungs is recognized and *Monilia* not infrequently are found as secondary invaders in lung abscess and other chronic pulmonary diseases. In primary infection, which appears to be more frequent in the tropics, chronic bronchitis or a more serious pneumonia is produced. Infection of the bones, joints, heart valves and meninges is rare.

*Candida* (*Monilia*) *albicans* is one of a number of species of yeast-like fungi which produces mycelium but no ascospores. Several species other than *C. albicans* have been associated with human moniliasis, although *C. albicans* appears to be the most frequent invader and is the only member of the group pathogenic for animals. Species are differentiated by cultural characteristics and fermentation reactions. Immunological studies have revealed a number of different types, but they do not serve as a basis of classification. In direct preparations from lesions the fungi are commonly seen as budding, yeast-like cells with or without mycelium. The fungus is gram-positive and is clearly seen in smears and tissues stained by this method. Cultures grow well on ordinary agar and broth or Sabouraud medium at room temperature of 37° C. Surface growth on solid media is white, creamy, dull and yeast-like. Young cultures are smooth, whereas older ones may develop surface irregularities or roughness, and extension into the agar may produce a bushy-appearing growth beneath the colony. Mycelial elements are, however, best observed in deep stab cultures or in cultures made by cutting into the surface of the agar with a straight inoculating needle.

The sensitivity of cultures of *Candida* to high dilutions of gentian violet forms the basis for the treatment of moniliasis of the skin and mucous membranes by local application of solutions of this dye. Other methods of value include application of other fungicidal and fungistatic preparations and occasionally x-ray therapy. Good personal hygiene, avoidance of excessive soaking and moisture of the skin and treatment of predisposing factors or disease are important. Therapy of systemic moniliasis is largely unsatisfactory.

### ASPERGILLOSIS

Human infection with members of the genus *Aspergillus* is rare, although the disease in animals is more frequent. Aspergilli are common saprophytic molds with world-wide distribution (Chapter 5) commonly found in soil and grain and on the skin as contaminants. The conidia or spores are frequently air-borne. Because of their wide distribution and frequent contamination it is often difficult to be certain of their pathogenicity. They may be present as secondary invaders in the cavities of tuberculosis of the lungs and in skin lesions. However, they may be found growing within the tissues, or in the absence of other microorganisms, so that primary aspergillosis is well recognized.

Aspergillosis is an important and fatal infection of the air sacs and lungs of domestic and wild birds, in whom the disease may be epidemic. It less frequently affects the lungs of mammals. Infection is usually traced to moldy grain.



may. In man aspergillosis is most frequently a superficial infection of the external ear, but it may involve the nasal passages, the lungs and, rarely, other parts of the body. Pulmonary aspergillosis closely resembles tuberculosis. Fragments of mycelium and spores are found in sputum and the fungi are found in tissues. Man may become infected by inhalation of spores from grain or from infected birds.

Although a number of species have been isolated from lesions of aspergillosis, *Aspergillus fumigatus* is most often cultured. Mycelium, often with aerial hyphae, conidiophores and conidia, are found in the tissues and typical colonies develop on culture media inoculated with infected material. Cultures grow rapidly on Sabouraud's agar at room temperature. *Aspergillus* may be recognized by the branched conidiophore which arises from a foot cell of the vegetative mycelium and terminates in a rounded vesicle. Primary and secondary sterigmata, which bear conidia in chains, arise from the entire surface of the vesicle. The conidia of *A. fumigatus* are green.

### SPOROTRICHOSIS

Sporotrichosis is an important fungus disease of man and animals caused by *Sporotrichum schenckii*. The fungus is world-wide in distribution and infection appears to be acquired from plants through contamination of injuries of the skin. Sometimes frequently infection may be acquired from patients or infected animals. Endogenous *Sporotrichum* has been recovered from plants and the disease appears to be relatively frequent among florists and agricultural workers. The most common form of the human disease involves the skin and lymphatic (lymph vessels and nodes) of the hand and arm. The lesions are chronic nodules which tend to ulcerate. It is unusual for the infection to become generalized, although lesions may be present over wide areas of the skin and mucous membranes.

*Sporotrichum schenckii* is observed with difficulty in direct preparations from lesions as gram-positive, spindle-shaped bodies and is more commonly demonstrated by cultivation or inoculation of male white rats. On Sabouraud's medium colonies are tough, tenacious, wrinkled, and cream to black in color. The mycelium is septate, branched and delicate ( $2\ \mu$  in diameter). Conidia are produced directly from the mycelium or in clusters from the tips of hyphae. Peritonitis and infection of the testes develop in rats following intraperitoneal inoculation of infectious material. In contrast to the human infection, numerous fungus cells may be demonstrated in gram-stained smears from the lesions in rats. Agglutination tests using patient's serum and a suspension of spores may be helpful in diagnosing the disease.

### THE ACTINOMYCETES—ACTINOMYCOSIS

The actinomycetes are a group of microorganisms transitional between the fungi and the bacteria, which are classified together with the mycobacteria, corynebacteria and *Streptomyces* in the order *Actinomycetales*. The diseases

produced by the pathogenic actinomycetes more closely resemble fungus bacterial infections and are hence considered with the mycoses.

An outline of the human diseases produced by the actinomycetes is given in Table 18. Members of the group are responsible for both superficial and deep infections, which are clinically and pathologically very different diseases and are caused by different actinomycetes. **Actinomycosis** is reserved for the granular disease typical of infection with *Actinomyces bovis* and *Nocardia asteroides* and does not refer to other diseases of the group. The actinomycetes also include in addition several pathogens of animals which do not affect man. Another of these organisms, which will not be discussed further, is *Actinobacillus lignihilus* of bovine actinobacillosis, and *Actinomyces farcinicus* of cattle farcy.

**Classification.** The relationships and general characteristics of the actinomycetes are given in Chapter 6. Subdivision of the group has, however, not been made according to several schemata. Use of terms, such as *Streptothrix*, *Corynebacterium*, and *Leptothrix* for members of the actinomycetes is now largely discontinued in favor of the general term *Actinomyces*. *Actinomyces* in the strict sense refers to the anaerobic or microaerophilic members of the group which produce branched mycelium and are not acid-fast. *Nocardia* differs in that its organisms are aerobic and may be acid-fast. Some authors prefer to use *Actinomyces* for the members of both groups.

The actinomycetes stain well with bacteriological dyes and are gram-positive. The mycelium is branched, approximately  $1\ \mu$  in diameter and nonseptate. Reproduction is by formation of conidia from the aerial mycelium or by fragmentation of the mycelium into bacillary arthrospores. The bacillary form predominates in cultures. The formation of enlarged or swollen club-shaped bodies arranged radially at the margin of the colony is typical of many cultures and gives the group its name of *Actinomyces* or "ray fungus." Although granules and vacuoles are sometimes seen, the internal structure of the actinomycetes generally is undifferentiated like that of the bacteria. Many species produce pigmented growth. Growth of most cultures occurs on bacteriological media and they utilize a wide variety of nutrient substances. Sabouraud's medium is satisfactory for growth.

**Actinomycosis (*Actinomyces Bovis*).** The name *Actinomyces bovis* was given by Harz to the ray fungus observed by Bollinger (1877) in pus of bovine actinomycosis or lumpy jaw and in the lesions of the human disease described one year later. Cultivation of fungi from actinomycosis has revealed that the essentially similar disease is produced by anaerobic or microaerophilic (*A. bovis*) and aerobic (*N. asteroides*) organisms.

Actinomycosis is world-wide in distribution, but it is a relatively uncommon disease. It is most frequently recognized in cattle, the estimated number of infected animals being somewhat less than 1 per cent of the total. Infection in other animals and man is infrequent. Relationship of the human disease to infection in animals and transmission of the infection by contact from one animal to another have not been established. *Actinomyces bovis* appears to



ctly parasitic and similar or identical organisms are normal inhabitants of mouth cavity of man and animals. The disease, therefore, probably represents invasion with organisms of the normal flora rather than a communicable infection. Human actinomycosis has been observed in all age groups, but most infections have been in young adults, particularly males. Injury, infection with other microorganisms and poor oral hygiene may be predisposing or contributing factors in some instances, although in others such conditions are not demonstrable. In animals injury of the mouth with hay or other food is frequent and vegetable particles are often found in the lesions of actinomycosis.



Fig. 173. Actinomycosis. Deeply staining granules (colonies) of the ray fungus are in the pus in an actinomycotic lesion. The granulation tissue wall of the lesion is above and below the colonies.

The lesions of actinomycosis resemble those of tuberculosis in that they consist of chronic inflammation with granuloma and abscess formation. Although *actinomyces* may invade any tissue of the body, including the skin, the deeper tissues and the bones, and tend to spread from one site of infection to adjacent tissues, invasion usually occurs through the oral cavity, the intestinal tract or the lungs. Deep infection frequently extends to and involves the skin, forming inflammatory sinus tracts which discharge pus. *Actinomyces* may be demonstrated in pus and infected tissues.

In animals actinomycosis usually involves the soft tissues and bony structures about the mouth, producing lumpy jaw. Infection of other regions of the body is infrequent and it is difficult to infect experimental animals with *A. bovis*. In man infection may occur in the tissues of the face and neck (cervico-facial actinomycosis), the lungs (pulmonary actinomycosis) or the intestinal tract and abdomen (abdominal actinomycosis). Cervico-facial infection, which is the most frequent form of human actinomycosis, appears to result from invasion from the mouth cavity. The soft tissues are primarily involved, the

bones usually remaining free of infection. The abdominal disease usually in the region of the appendix and results in abscess formation in the abdominal cavity, often with spread to the liver. Pulmonary actinomycosis resembles tuberculosis in many instances, although a more acute type of disease may simulate pneumonia. Infection in other tissues of the body appears to be secondary to these primary types of actinomycosis.

In pus and tissues infected with *A. bovis*, the fungi are observed in yellowish granules, known as **sulfur granules**, which are in fact colonies composed of tangled filaments with peripheral clubs radiating like the spokes of a wheel from the central mass. The aerobic *Actinomyces* (*Nocardia*) also produce this typical colonial structure but are seen as tangled filaments in the lesions. *Actinomyces bovis* is anaerobic or microaerophilic and is cultivated with difficulty on enriched bacteriological media. The colonies are small, regular, white or yellowish in color and resemble colonies of bacteria. Branching filaments, fragmented mycelium and rods are seen microscopically. *Nocardia* grows aerobically, producing wrinkled, irregular colonies, which microscopically are composed of typical *Actinomyces*. The diagnosis is made by the observation of typical gram-positive organisms in material from lesions and by cultivation.

The physiology and immunology of the *Actinomyces* are poorly known. The treatment of the disease prior to use of the sulfonamide drugs and penicillin was unsatisfactory. The latter drugs, however, appear to be effective in many cases.

**Mycetoma.** Mycetoma (maduromycosis, Madura foot) is a chronic fungal infection of the lower extremities, which occurs principally in tropical and subtropical areas where people do not wear shoes and injury of the feet is common. The disease is similar to actinomycosis and, indeed, *Actinomyces* or *Nocardia* are found in a high proportion of infections. A variety of other fungi, many of questionable significance, have also been observed. Infection is thought to be exogenous and to occur through sites of injury.

**Erysipeloid** (*Erysipelothrix rhusiopathiae*). *Erysipelothrix* is a gram-positive, filamentous bacillus which closely resembles the actinomycetes. The organism is microaerophilic and produces minute, colorless, smooth or wrinkled colonies. The organism is primarily a parasite of lower animals, and human infection (erysipeloid) is almost invariably acquired from animal sources. The infection of swine is best known and is highly fatal. In these animals the disease occurs in several forms (swine erysipelas, diamond skin disease, arthritis, septicemia) and the organisms are found in excreta. Human infection is an occupational disease of agricultural workers, fishermen, veterinarians and others who handle infected animals or their products. Infection of the hand and arm resembling erysipelas of streptococcal etiology is the most frequent form of human disease, although more severe infection sometimes occurs.

**Actinomycotic Rat-Bite Fever and Haverhill Fever.** *Streptobacillus moniliformis* (*Actinomyces muris-ratti*, *Haverhillia multififormis*) is a non-



philic, gram-negative, pleomorphic and filamentous microorganism, which appears to be a natural parasite and pathogen of rats and mice. Enriched media required for growth and carbon dioxide is beneficial. In rodents, the organism is commonly found in the nasopharynx and is the cause of bronchopneumonia, bacteremia and infection of the joints in these animals. Human infection is rare and may be acquired through rat bite, although not all cases are acquired in this way. Indeed, the epidemic form of the disease (Haverhill fever) appears to be milk-borne. Infected persons develop severe malaise, arthritis and a skin rash which resembles measles. Following rat bite the site of the injury becomes inflamed before development of the typical symptoms. The causative organism can be isolated from the blood and tissues.

### BLASTOMYCOSIS—BLASTOMYCES DERMATITIDIS

American blastomycosis (Gilchrist's disease), caused by *Blastomyces dermatitidis*, was first described by Gilchrist in 1894 and is one of the more frequent and important fungus infections. The disease appears to be limited in geographic distribution to North America and is most often recognized in the Mississippi Valley. American blastomycosis is to be distinguished from infection with *Cryptococcus* (cryptococcosis, torulosis, European blastomycosis).

Blastomycosis may affect either the skin (cutaneous type) or the deep tissues (systemic or disseminated type). Infection in the former usually occurs through the skin and in the latter via the respiratory tract. The sources of infection with *dermatitidis* are poorly known, but person-to-person transmission occurs infrequently or after prolonged contact. The distribution of lesions and low communicability point to some exogenous source, presumably an animal reservoir. Infection of animals has, however, seldom been demonstrated and the fungus has not been cultivated from the soil or plants.

Cutaneous blastomycosis invades exposed areas of skin (face, hands, arms, feet or extremities) and, although spread of infection to tissues adjacent to the original location is the rule, there is little tendency for the disease to become disseminated. The skin lesions are dull red areas of chronic granulomatous inflammation which often have a crusted or warty surface. Small abscesses in the skin and subcutaneous tissues contain *Blastomyces*. There is a tendency for the central portion of the lesion to heal while there is extension of the disease at the margins.

Systemic blastomycosis is a relatively prolonged infection which clinically resembles advanced or miliary tuberculosis. The lungs are the site of a progressive pneumonia or abscess, and as the disease advances other organs, the skeleton, skin and the central nervous system are frequently infected. Microscopically the lesions are those of a granuloma with small abscesses containing the fungi. Diagnosis is made by demonstration of the fungi, although the blastomycin skin reaction and immunological tests may be valuable aids. Cutaneous blastomycosis

cosis is seldom fatal, but it is a prolonged infection. Systemic infection the other hand, is usually fatal. *Blastomyces* is only slightly pathogenic to animals.

*Blastomyces dermatitidis* is present within infected tissues as thick-walled yeast-like organisms which occur singly or as budding organisms, a point of differential significance since *Coccidioides* reproduces by formation of endospores. The organisms may be observed in tissue sections or in direct preparation of pus, skin scrapings or sputum. Cultures develop on blood agar and on Sabouraud's

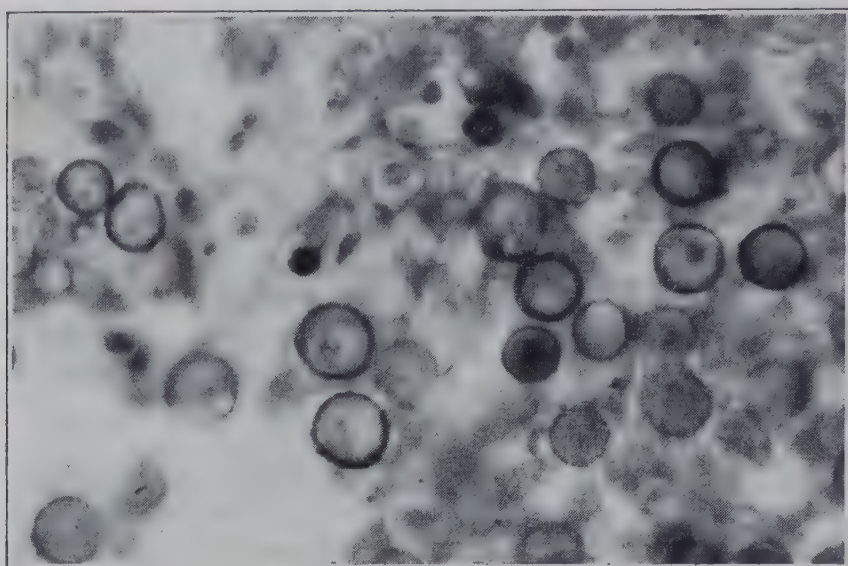


Fig. 174. American blastomycosis. Thick-walled blastomyces are seen within lung in pulmonary blastomycosis. Note the budding form near the top. (Magnification approximately  $\times 750$ .) (Courtesy of Dr. E. M. Humphreys.)

medium at room or body temperature. At room temperature on Sabouraud's medium a typical white, cottony, fungus type of colony composed of septate hyphae with conidia on lateral conidiophores is produced. In freshly isolated cultures or on blood agar at  $37^{\circ}\text{C}$  the growth is, on the other hand, waxy and composed of yeast-like cells with only fragments of mycelium. Specific complement-fixation is obtained with patient's serum and *B. dermatitidis* in systemic blastomycosis, but the reaction is inconstant in superficial infection. Immunity is, however, ineffective against the disease. Specific hypersensitivity may be demonstrated by the intracutaneous inoculation of a killed suspension of whole cells or cellular extracts.

### CRYPTOCOCCOSIS—CRYPTOCOCCUS NEOFORMANS

Cryptococcosis (torulosis, European blastomycosis) is an infection with *Cryptococcus neoformans* (*Torula histolytica*), described in 1893 by Busse and Buschke. The disease is infrequently recognized, but it has a wide geographical distribution, including Europe, North and South America, Australia and the Pacific area. Although cryptococcosis is a disease of many parts of the body,



fection of the brain and meninges (*Torula meningitis*) is particularly serious and is the form most frequently recognized in the United States. In the type of infection known as European blastomycosis abscesses and nodules containing fungi are found in the skin, the lymphatics, the deep tissues and bones. Acute pulmonary cryptococcosis is a not uncommon form. In *Torula meningitis* the symptoms are those of slowly developing, progressive central nervous system disease (headache, vomiting, mental disturbances, delirium and coma). There is usually little fever and the neurological findings are those of intracranial pressure and chronic infection. The infection involves primarily the meninges, but lesions within the brain substance and along the blood vessels may be present. The insidious nature and infrequent recognition of the disease make epidemiological study difficult. However, recent evidence suggests that human infection and disease are more frequent than earlier evidence indicated. Within the tissues and the spinal fluid *Cryptococcus neoformans* appears as single or budding encapsulated, thick-walled yeast-like cells. In cultures at room or body temperature the growth is white, wrinkled and yeast-like or mucoid. The fungus reproduces primarily by budding in culture as well as in the body, although short germination tubes (rudimentary mycelium) may be observed and ascospores have been reported. The diagnosis is made by observation of the typical fungi in direct preparations, cultivation and tests for pathogenicity in mice.

Treatment of systemic cryptococcosis has until recently been largely unsatisfactory. However, somewhat encouraging results have been obtained with diamide drugs.

### COCCIDIOIDOMYCOSIS—COCCIDIOIDES IMMITIS

Coccidioidomycosis (coccidioidal granuloma) is a fungus disease caused by *Coccidioides immitis*. Although the disease has been reported elsewhere, infection is rare outside the southwestern United States, where it is endemic. The majority of cases is reported from the San Joaquin Valley in California (valley fever, desert rheumatism). Within the endemic area, a benign or primary form of the disease is frequent and coccidioidin tests indicate almost universal infection of the population. The disease affects all age groups, but it is particularly frequent among young adult males. *Coccidioides* has been recovered from domestic animals, but human infection appears to be acquired by inhalation of spores of the fungus in dust or by invasion through the mouth and skin. *Coccidioides* has been isolated from the soil and from native small rodents, which may be responsible for contamination of the soil. Laboratory infection with spores from cultures may occur, so that careful technique must be employed.

Coccidioidomycosis occurs as either a self-limited primary disease or as a progressive systemic coccidioidal granuloma. The primary infection is usually a pneumonia, the lung lesions of which either resemble bacterial pneumonia or are nodular, frequently with cavitation as in tuberculosis. There is enlarge-

ment of the thoracic lymph nodes in some cases. Primary coccidioidomycosis of the skin and the lymph nodes of the neck is also recognized. Arthritis, erythematous skin rash, which are considered to be allergic in nature, occasionally appear a short time after primary infection. Usually primary coccidioidomycosis subsides spontaneously and many subclinical or unrecognized mild infections undoubtedly occur. Progressive or generalized infection is an infrequent fatal disease. There is extensive infection of the lungs and invasion of the bones, the viscera and often the meninges, with fever, difficulty in breathing and progressive loss of weight and strength.

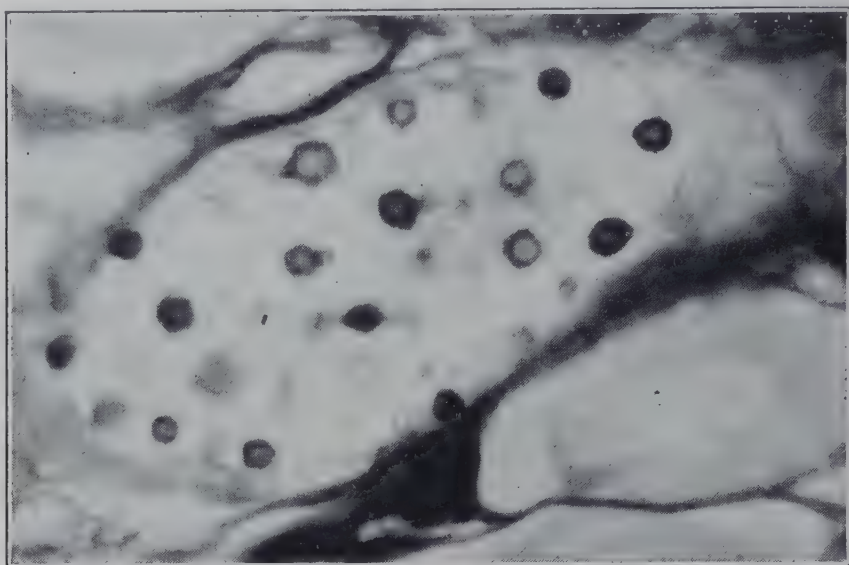


Fig. 175. Cryptococcosis. Fungi, several of which are budding, are seen within a tubule of the kidney. Note the thick wall of the fungi and the clear areas representing the capsule surrounding many of the cells. (Magnification approximately  $\times 750$ .) (Courtesy of Dr. E. M. Humphreys.)

*Coccidioides immitis* appears in the tissues as thick-walled, spherical (20 to 80  $\mu$  in diameter) which are filled with endospores (approximately 2  $\mu$  in diameter). Fungi may also be found in sputum, pus and spinal fluid on microscopic examination or may be demonstrated by cultivation and inoculation of guinea pigs or mice. *Coccidioides* may be cultivated on Sabouraud's agar at room temperature. Colonies are cottony or smooth and waxy and white to brown in color. Septate mycelium and conidia or arthrospores are observed microscopically.

The hypersensitivity test has the same significance as the tuberculin reaction and is performed by inoculating intracutaneously 0.1 ml. of a 1:100 dilution of coccidioidin (a standardized sterile filtrate of a liquid culture of the fungus). Immunity is manifest by the frequency of subclinical and primary infections which subside spontaneously. Immune reactions other than hypersensitivity reactions, however, demonstrated with difficulty. There is no specific treatment for the disease.



Histoplasmosis was originally described by Darling (1906) as a disease similar to kala-azar. The causative agent was not isolated until 1934 (DeMonbreun) at which time its fungus nature was established. For some years following its discovery histoplasmosis was infrequently recognized and was considered to be a rare disease. In recent years, however, increasing frequency of identification of the fungus within the tissues and the results of skin tests with histoplasmin have shown that infection is more widespread than was formerly thought to be the case. Recent surveys in the United States using histoplasmin have indicated

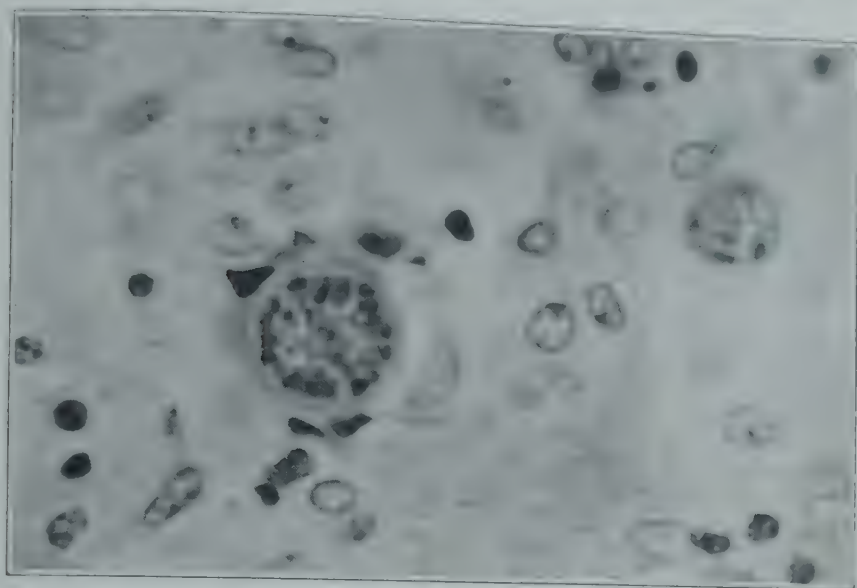


Fig. 176. Coccidioidomycosis. Two large sporulating fungi are shown in the granular tissue of infected lung. Note the small spores within the large cyst and the thick, refractile wall. (Magnification approximately  $\times 750$ .) (Courtesy of Dr. George Mori.)

that hypersensitivity is relatively frequent in the general population, particularly in the middle western area and that a positive skin test has the same significance as does the tuberculin test in tuberculosis surveys. Furthermore, in some areas there is some correlation between reactivity to histoplasmin and x-ray evidence of otherwise unrecognized and calcified lesions of the chest. Histoplasmosis, therefore, must be considered along with tuberculosis as a cause of pulmonary pathology. Present evidence indicates that infection with *H. capsulatum* has a wide geographic distribution and that all age groups are affected, but that the disease is more frequent among children and older adults. The incidence is higher among males than females. Infection is presumably exogenous and naturally infected dogs and rats have been found. Additional evidence is, however, necessary to establish the epidemiology of infection with *Histoplasma*.

Disease caused by *H. capsulatum* is of several types. Infection may be primarily of the lymphoid-macrophage system, producing a febrile disease with enlargement of the liver and spleen. The lymph nodes (glands) may be generally enlarged. In other instances ulceration of the skin or intestinal tract may be dominant and, although infrequently recognized clinically, infection of the

lung would appear from x-ray and skin test evidence to be a frequent focus of infection. Active pulmonary disease resembles tuberculosis. Accumulating evidence indicates that any tissue of the body may become infected. The route of entrance of *H. capsulatum* into the body is not well known. However, the respiratory tract and the gastro-intestinal tract appear to be the most probable points of entry.

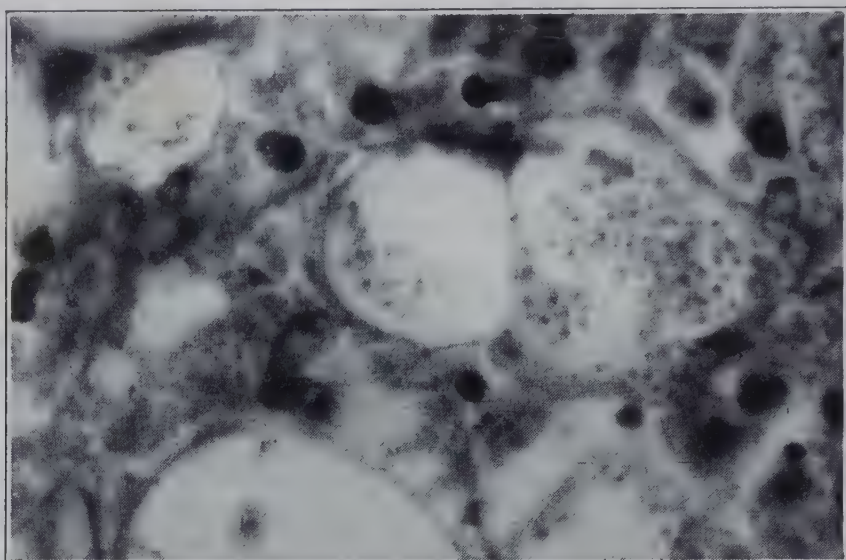


Fig. 177. Histoplasmosis. Many small fungi are seen within the macrophages of bone marrow. (Magnification approximately  $\times 750$ .) (Courtesy of Dr. E. Humphreys.)

The organisms are found within the macrophages in the spleen, liver, bone marrow and in tissue cells and inflammatory nodules elsewhere. Within tissues the organisms are small (approximately  $5 \mu$ ), encapsulated, round or oval cells. In culture round and oval budding yeast-like cells are also seen and septate mycelium is produced at room temperature. Conidia and thick-walled chlamydo-spores, which have spinous, tuberculate projections, are produced. Colonies of the yeast-like phase are white, moist and yeast-like, whereas those of the mycelial type are white to brown in color and cottony in appearance. Laboratory animals may be infected experimentally.

Complement-fixing antibodies and hypersensitivity to culture filtrates have been demonstrated and are of diagnostic significance. The skin-testing antigen is a sterile filtrate of a three-months-old culture on a synthetic liquid medium. Following intracutaneous inoculation of 0.1 ml. of a 1:1000 dilution of antigen either an immediate or delayed tuberculin-type reaction is obtained. Cross-reactions occur with *Blastomyces* antigens.



## PROTOZOA OF MEDICAL IMPORTANCE

Parasitic and pathogenic Protozoa have been recognized in four classes, *Sarcodina*, *Infusoria*, *Sporozoa* and *Mastigophora* (Chapter 3). These infections include some of the most important diseases of man, for example malaria, amebic dysentery, African sleeping sickness, oriental sore and kala azar. The important man parasites of these classes, the diseases which they produce, lower animal hosts and location of the parasites within the body are shown in the accompanying table. Because of the larger size, their complex structure, the difficulty in cultivation and in many instances the host specificity and complex life cycle of the parasitic Protozoa, the methods for studying them are different from those applied to the other microorganisms. The physiology and immunology of these organisms are, therefore, relatively poorly known, whereas morphology and epidemiology have received particular attention.

### SARCODINA—THE PARASITIC AMOEBAE

Amoebae are common parasites of man and are world-wide in distribution. From 25 to 50 per cent of the population are found to harbor the nonpathogenic *Endamoeba coli* and *E. gingivalis*, and approximately 10 per cent are infected with the pathogenic *E. histolytica*. *Endolimax nana* and *Iodamoeba williamsi* are also common parasites, whereas *Dientamoeba fragilis* is rarely identified. Amoebae are most frequent in tropical regions and in areas where sanitation is poor.

The genera are differentiated by their nuclear structure. The nucleus of *Endamoeba* has a marginal layer of chromatin at the nuclear membrane, a small karyosome and a delicate linin network. The karyosome is large in the nucleus of *Endolimax*, and there is no chromatin layer in the nuclear membrane. In *Iodamoeba* the karyosome is large and surrounded by poorly staining globules and a delicate linin network. *Dientamoeba* has two nuclei, the chromatin of which is collected in four granules. *Dientamoeba* does not form cysts.

**Endamoeba Histolytica—Amebiasis.** *Endamoeba histolytica* was probably first recognized by Lösch in 1873, and was definitely associated with amebic dysentery by Koch and Gaffky (1883). Since these early descriptions, amebic dysentery has been recognized throughout the world, but it is most frequent in tropical climates. Although the intestinal tract is the initial and most common site of infection, amebic abscesses have been found in the liver and less fre-

TABLE 19. THE PARASITIC AND PATHOGENIC PROTOZOA OF MAN

PARASITE	DISEASE	LOWER ANIMAL HOST	LOCATION OF PARASITE IN THE HUMAN BODY
CLASS: SARCODINA <i>Endamoeba histolytica</i>	Amebiasis (amebic dysentery)	Cats, dogs, rats and monkeys may be naturally infected	Intestinal tract, usually intestine; abscesses elsewhere, particularly the liver
<i>Endamoeba coli</i> , <i>Endolimax nana</i> , <i>Iodamoeba williamsi</i> (bütschlii), <i>Dientamoeba fragilis</i>	Not pathogenic		Lumen of intestinal tract
<i>Endamoeba gingivalis</i>	May be found in gingivitis and abscesses		Oral cavity; particularly about the teeth in gingivitis and pyorrhea
CLASS: INFUSORIA (CILIATA) <i>Balantidium coli</i>	Balantidiasis (ciliate dysentery)	Pigs; similar parasites in other lower animals	Intestinal tract; usually intestine
CLASS: SPOROZOA <i>Plasmodium vivax</i> <i>Plasmodium malariae</i> <i>Plasmodium falciparum</i> <i>Plasmodium ovale</i>	Human malaria	Anopheline mosquitoes	Intracellular parasites of red blood cells, possibly of macrophages in some stages
<i>Toxoplasma capsulatum</i>	Toxoplasmosis		Brain in encephalitis; lungs in pneumonia
SARCOSPORIDIA ( <i>Sarcocystis</i> spp.)	Rarely affects man	Many vertebrates	Muscles
<i>Isospora hominis</i>	Rare	Many vertebrates harbor related parasites	Intestinal tract; cells of mucosa
CLASS: MASTIGOPHORA <i>Chilomastix mesnili</i> <i>Giardia lamblia</i>	Possibly diarrhea	Many vertebrates harbor similar parasites	Intestinal tract; lumen of intestinal tract
<i>Trichomonas</i> spp. ( <i>hominis</i> , <i>vaginalis</i> , <i>buccalis</i> )	vaginitis; possibly diarrhea		Intestinal tract; female genital tract; mouth
<i>Trypanosoma gambiense</i> and <i>T. rhodesiense</i>	African sleeping sickness	Tsetse fly ( <i>Glossina palpalis</i> , <i>Glossina morsitans</i> )	Many tissues, particularly blood and central nervous system
<i>Trypanosoma cruzi</i>	Chagas' disease	Bugs: <i>Triatoma megista</i>	Tissues and blood
<i>Leishmania donovani</i>	Kala-azar	<i>Phlebotomus</i> spp. sand flies	Tissues; particularly liver, spleen and bone marrow
<i>Leishmania tropica</i>	Oriental sore	<i>Phlebotomus</i> flies	Tissue cells of the skin lesions
<i>Leishmania brasiliensis</i>	South American leishmaniasis	<i>Phlebotomus</i> flies (?)	Tissue cells of skin lesions



ntly in the lung, brain and spleen. Asymptomatic carriers of infection are recognized and natural infection of dogs, cats, rats and monkeys has been reported. However, man is the principal natural host, and it is doubtful if lower animals are important in the epidemiology of the disease. Kittens may be used in experimental studies. Other laboratory animals have at times been infected.

**Morphology and Life Cycle.** The size of the active stage or trophozoite of *E. histolytica* is variable, but it is usually between 20 and 30  $\mu$ . The cell wall is thin and there is a thick layer of clear ectoplasm surrounding the granular cytoplasm. Movement is accomplished by the rapid extension of pseudopodia of ectoplasm from any part of the cell surface, so that the cells are often irregular in shape. The cytoplasm is seen to contain food vacuoles filled with red blood cells from the host. The nucleus has a delicate appearance with a thin layer of chromatin and a small centrally placed karyosome. Multiplication is by binary division and by cyst formation. In the precystic stage the cells are round and usually have no food vacuoles. The cyst is spherical with a thin but resistant membrane and when mature contains four nuclei and blunt, rod-shaped tomontoid bodies. Active trophozoites are usually observed in acute infections. Tomontoid cysts predominate in carriers.

The cyst of *Endamoeba histolytica* is relatively resistant to adverse temperatures and to disinfectants, but is readily destroyed by heat or drying. In contaminated water they may remain viable for many days and are the infective stage of the parasite. The only known way of acquiring infection is by ingestion of the viable cysts. Flies and other animals may play a role in dissemination of cysts from unprotected latrines, and contaminated water and food are important means of transmission.

Excystation with development of trophozoites occurs in the intestinal tract of a new host or under suitable conditions of temperature and moisture elsewhere. The single large multinucleate cell (four nuclei in cyst stage) rapidly produces many amoebae by division of the nuclei and cytoplasm.

**Physiology.** Within the host the nutrients of *E. histolytica* consists of red blood cells and of the host tissues which are destroyed by the amoebae. In cultures the amoebae ingest bacteria and other food particles and may not be able to live in the absence of such food particles.

**Pathogenesis.** Following ingestion of parasites by the host and excystation the amoebae become established in the wall of the large intestine. The cecum, ascending colon and lower sigmoid or rectal regions are most commonly invaded, although any part of the large intestine may be infected. Injury of the tissues may be sufficiently severe to produce an abscess followed by ulceration, bleeding and dysentery or may be less severe and asymptomatic. Erosion of small blood vessels of the intestinal wall permits access of the amoebae to the blood and access to the liver and other tissues in which abscesses may develop. The intestinal lesions show intense inflammation and reparative processes with scarring. Amoebae are found deep within the lesions at the border of the necrotic central portion of the ulcer with the living tissues of the host. It is not known

with certainty in asymptomatic infections if the amoebae live as commensals within the lumen of the intestine or produce tissue injury that is repaired by the host. Chronic amebiasis with lesions and symptoms is recognized.

The relationships between *E. histolytica* and its host are complex and the mechanisms of virulence are obscure. Differences in virulence between strains may be a factor, but the same strain may give rise to acute dysentery in one individual and a latent infection in another individual.

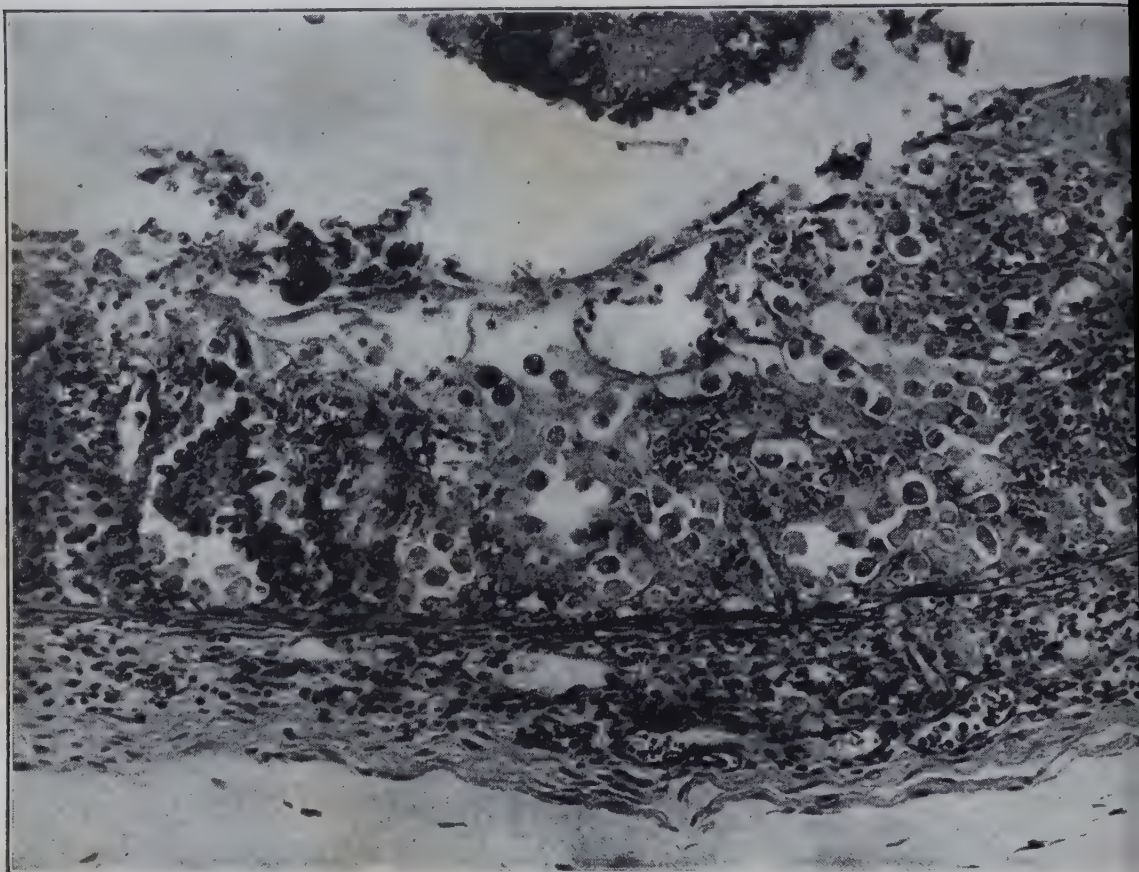


Fig. 178. Tissue section of amebic ulcer of the intestine. Large numbers of amoebae are seen invading the intestinal wall (approximately  $\times 100$ ). (From Duval and Schenck: *Textbook of Pathology*, and Belding: *Textbook of Clinical Parasitology*, D. Appleton-Century Co., Inc.)

**Diagnosis.** The diagnosis of infection with *E. histolytica* is made by demonstration of the organisms in stool specimens. As indicated above, trophozoites are more often recognized in the acute disease and cysts are characteristic of chronic or asymptomatic infections. It is important to examine fresh, watery stool specimens or material obtained at proctoscopic examination in order to detect ameboid motion of the living trophozoite, which rapidly becomes inactive upon cooling. The organisms must be differentiated morphologically from *E. coli*. The latter organism is similar in size and general characteristics to *E. histolytica*. The food vacuoles, however, contain bacteria and other materials, but no blood cells. The nuclear chromatin is more abundant and the karyosome is



ric in position. The cyst stage usually contains eight nuclei and the chromobars are splinter-shaped. Cysts are best detected in dilute iodine solution. Concentration by flotation in zinc sulfate solution may be of aid. Cultivation methods may also be used in diagnosis.

**Immunity.** Complement-fixing antibodies may be demonstrated in a high proportion of persons infected with *E. histolytica*. Resistance to infection appears to be largely a matter of cellular inflammatory response with localization of infection and repair of injured tissues. There is little or no evidence of effective acquired immunity. Treatment is by the use of drugs, chiefly emetine, vioform, and chiniofon.

## INFUSORIA

### *Ciliata*

**Balantidium Coli.** Although lower animals have many parasitic ciliates, none of very complex structure, the only species parasitic in man is *Balantidium coli*. *Balantidium coli* is a larger parasite, approximately 60  $\mu$  in length and 50  $\mu$  in width. It is actively motile by means of short cilia which cover the surface of the oval-shaped cell. There are two nuclei, a macronucleus which is vegetative and a micronucleus which functions in sexual reproduction or conjugation of the trophozoan. Food particles (bacteria, red and white blood cells, starch, etc.) are taken into the peristome by ciliary action, enter food vacuoles in the cytoplasm, are digested by the cellular enzymes. Débris is extruded from the cell through the excretory cytopye. Several contractile vacuoles dispose of soluble waste products. The trophozoites reproduce by binary fission and by conjugation. The cyst has a thick, resistant wall and is approximately 50  $\mu$  in diameter.

*Balantidium coli* is an intestinal parasite acquired by ingestion of contaminated food and water. Cysts and possibly trophozoites are infectious. The pig is the natural host of *B. coli* and human infections appear to be acquired from this animal. It is interesting in this connection that cysts are more commonly observed in porcine than in human infections. Balantidia are also commonly found in the guinea pig and several primates other than man. Balantidia are found in the cecum and adjacent ascending colon. Human infection is accompanied with diarrhea or dysentery in the acute stages. The parasites invade the intestinal wall, producing small abscesses and inflammation similar to amebic dysentery. In other instances infection is asymptomatic and carrier-like. Diagnosis is made by demonstration of the parasite or cyst in fresh stool specimens. Treatment by chemotherapy with carbarsone or other parasiticides is partially satisfactory and many infections are eliminated spontaneously, although severe infection may be fatal.

## SPOROZOA

**The Malarial Parasites.** Malaria, which is a disease characterized by regularly intermittent chills and fever, has been recognized since antiquity and remains the most important protozoal disease if not the most important infec-

tious disease of man. The name **malaria** is derived from association of disease with swamps and marshes and the belief that it was acquired from bad (*mal*) air (*aria*) of these regions. The malarial parasites were first described by Laveran (1880) and the life cycle in mosquitoes was demonstrated by Sir Ronald Ross (1898) for bird malaria and by Grassi (1898) for the human disease. The asexual cycle in man was described by Golgi, who also separated the different species of human parasites.

Four species of human malarial parasites are recognized, *Plasmodium vivax*, *P. malariae*, *P. falciparum* and *P. ovale*. These parasites have complicated cycles which are essentially similar. Within the human body the parasites multiply asexually by segmentation into many progeny, and in the mosquito by sexual reproduction with production of many infective parasites. Malaria is acquired naturally only by the bite of infected mosquitoes.

**Life Cycle.** The infective stage of the parasite received by man from a mosquito is an elongated, spindle-shaped parasite 10–12  $\mu$  in length known as a **sporozoite**. Formerly thought to parasitize the red blood cells directly, experimental work with animal malaria has demonstrated that the sporozoites enter tissue macrophages and there produce by segmentation many small daughter cells or **cryptozoites**. The cryptozoites are able to penetrate the red blood cells and establish the blood stream infection. The infection is then maintained in the human host by the **asexual cycle**. After invasion of the erythrocyte the parasite (trophozoite) goes through a period of growth from the initial small **ring stage** to the larger **schizont** and **segmenter** (Fig. 178). By a series of divisions, first of the nucleus and then of the cytoplasm, the segmenter produces daughter cells which, upon rupture of the red blood cell, become free **merozoites** and parasitize fresh red blood cells. The time required for completion of the asexual cycle varies between 48 and 72 hours and is constant for the different types of malarial parasites. The correspondence between the clinical chills and fever with the production of successive broods of merozoites gives malaria its tertian (48-hour) or quartan (72-hour) periodicity.

The **sexual cycle** of the malarial parasite begins in the human host but must be completed in the mosquito. The sexual stages within the blood (**gametocytes**) do not contribute to the production of disease within the same individual, but rather are responsible for transmission by the mosquito to new hosts. Gametocytes are produced from certain of the trophozoites, which in the early stages of development are not distinguishable from parasites of the schizont series. Later, however, **macrogametocytes**, the female sexual cells, and **microgametocytes**, the male sexual cells, are morphologically recognizable. Within the mosquito's stomach, the mature macrogamete is fertilized by one microgamete which is an actively motile, spindle-shaped parasite produced from the microgametocyte by a process known as exflagellation. The fertilized cell, or **zygote**, develops into a motile form, the **ookinete**, which penetrates the epithelium of the mosquito stomach and forms a cyst (oocyst) in the outer coats of the stomach wall. The parasite undergoes rapid development and produces many





Fig. 179. Diagram of the (1) ring, (2) schizont, (3) segmenter and (4) gametocyte: stages in the life cycle of (A) *Plasmodium vivax*, (B) *P. Malariae* and (C) *P. falciparum*. (After Hegner and Cort, from Hegner, Root, Augustine and Huff: *Parasitology*, Appleton-Century Co., Inc.)

hundreds of sporozoites. Upon rupture of the oocyst, which occurs within a few days, the sporozoites are liberated into the body tissues of the mosquito. Those of importance in the transmission of malaria migrate to the salivary glands and are inoculated into the new host by the mosquito bite.

***Plasmodium Vivax.*** All stages of the life cycle of *P. vivax* are observed in the peripheral blood. The ring stage is not distinctive. The early trophozoite is an irregular ameboid cell. As the parasite grows, the red cell becomes enlarged and there is accumulation of granular debris of hemoglobin digestion and pinpoints to red staining dots known as Schüffner's dots. An average of 16 merozoites are produced from each segmenter. The macrogametocytes (11 to 16  $\mu$  in diameter) have a deeply staining cytoplasm. There is a single mass of nuclear material and rod-shaped pigment. The microgametocyte is a smaller cell with lightly staining cytoplasm and less compact or granular nuclear material. The asexual cycle of *P. vivax* is completed in 48 hours, thus producing tertian bouts of fever ("tertian" since the day of fever was counted as day one, the intervening day as day two, so that the next bout of fever occurred on day three). The disease is known as benign tertian malaria.

***Plasmodium Ovale.*** *Plasmodium ovale* resembles *P. vivax* in all essential details and is often considered to be a race of the latter parasite. Its name results from the oval shape of the parasitized cells.

***Plasmodium Malariae.*** *Plasmodium malariae* produces no enlargement of the red blood cell, Schüffner's dots are absent and the parasites are generally smaller than *P. vivax*. The young trophozoite is more rectangular or band-shaped than ameboid and irregular. The 6 to 12 merozoites of each segmenter are usually arranged in the form of a rosette. The gametocytes are less pigmented. The asexual cycle of *P. malariae* is quartan, i.e., 72 hours are required for completion. All stages are found in the peripheral blood.

***Plasmodium Falciparum.*** *Plasmodium falciparum* is distinguished by the delicacy of the ring stage, the crescent shape of the gametocytes and the frequency of multiple parasites within a single red blood cell. Since schizogony takes place within the capillaries of the deep tissues, other forms of the parasite are seldom seen in the peripheral blood. Each segmenter produces approximately 10 merozoites in a 36- to 48-hour cycle. Infected red blood cells are not enlarged and contain large pigment granules known as Maurer's dots.

***Epidemiology.*** With the exception of a few isolated areas, malaria is present in the tropical and subtropical regions (60° N. to 30° S. latitude) throughout the world. *Plasmodium vivax* is the most widespread parasite, *P. malariae* and *P. falciparum* being more limited in distribution. The latter species is, however, predominant in most tropical regions. The human malarial parasites are all transmitted in nature by anopheline mosquitoes. Only the female mosquito bites and, hence, is the vector of malaria. Of the many recognized species of anopheline mosquitoes only a few are important vectors of human malaria, and prevalence of the disease is in part determined by the density of the population of effective anopheline vectors. These mosquitoes are present in large numbers in highly



various regions and habitually feed on human blood. The female mosquito becomes infected following a meal of blood containing parasites and, after the incubation period required for maturation of the sporozoites, transmits the parasite at subsequent feedings. Malaria may be exceeded as a cause of disability and death among the infectious diseases only by tuberculosis. Malaria is the cause of over 1,000,000 deaths per year, widespread chronic disability, economic and social retardation. The incidence of malaria is greatly affected by temperature, moisture, topography and season. It occurs in the lowlands where high temperature and warm temperatures favor the breeding and activity of mosquitoes. It is rare in mountainous regions and in dry seasons. In semitropical regions the disease is particularly prevalent in spring and autumn.

**Pathogenicity.** The human malarial parasites infect only man; there are no other animal reservoirs. Conversely, with the exception of one monkey parasite, man does not become infected with the malarial parasites of lower animals. Following infection there is an incubation period of 9 to 21 days before the onset of symptoms. Aestivo-autumnal malaria (*P. falciparum*) has a short incubation period, that of benign tertian (*P. vivax*) is intermediate and quartan malaria (*P. malariae*) is relatively slow in development. Symptoms do not begin until considerable numbers of parasites, estimated at 150,000,000, have been produced by schizogony. The characteristic symptoms of malaria are the regularly recurrent attacks of chills followed by fever with malaise, weakness, prostration, headache and sweating. The fever is at first irregular but in single attacks it soon assumes its tertian or quartan character, although multiple infections with different species or broods of the same species may give daily febrile episodes. An anemia, which may be severe, develops as the disease progresses and the spleen becomes enlarged and engorged with blood. The macrophages of the liver, spleen and bone marrow are found filled with parasitized and partly destroyed erythrocytes and red cell debris. During the acute phase parasites are numerous in the blood and masses of parasitized cells may be found in the small capillaries, particularly in infections with *P. falciparum*. Infection of the brain with coma and collapse, intense destruction of red blood cells with hemoglobinuria (blackwater fever) and especially heavy infection of the blood are not infrequent in this malignant type of the disease. The brain may be damaged by lodgement of parasitized cells in the small cerebral vessels. In nonfatal infections the acute attack subsides into a latent or chronic and recurrent infection after several weeks. Relapse is frequent, particularly during the first few years after the initial attack. Parasites are not demonstrable by laboratory methods during latency, but blood may be infectious for mosquitoes during chronic infections and malaria may be transmitted by transfusion.

**Diagnosis.** The specific diagnosis of malaria is made by demonstrating parasites in the peripheral blood. Their presence is conveniently detected by the examination of thick smears in which the red blood cells are laked or destroyed by staining without fixation, but the identification of the species of *Plasmodium* is

made by study of thin blood films. Periodicity of the fever, splenic enlargement and response to antimalarial drugs are of aid.

In surveys to detect the amount of disease within the community and chronic infections, parasites may be difficult to find in blood smears. In some instances, particularly in surveys, the determination of the spleen index or percentage of people having enlarged spleens is valuable.

**Treatment.** Malaria is treated by chemotherapeutic agents, the most honored of which is quinine, although the dyes atabrine and plasmochin are also well established. Both quinine and atabrine act against the trophozoites and hence tend to reduce the total numbers of parasites. These drugs are about equally effective and neither is active against gametocytes. They may be used in the treatment of acute malaria or in small doses as suppressive drugs to maintain the numbers of parasites at a low level, thus preventing development of symptoms. Plasmochin is particularly active against gametocytes and has more value in *P. falciparum* infections. Plasmochin is toxic and hence must be used with caution. Complete elimination of malarial infection is difficult to accomplish with any of these drugs, so that relapse is not infrequent following treatment. Indeed, the perfect antimalarial drug has not been discovered, although some of the newly introduced drugs, such as chloroquine and pentaquine, appear to be exceptionally effective.

**Immunity.** Immunity in malaria is primarily cellular immunity, dependent upon activity of the macrophages, particularly those of the liver, spleen and bone marrow. These cells engulf and destroy tremendous numbers of parasites and red blood cells during the initial infection and their activity is increased by acquiring immunity during the later part of the initial attack and in chronic infections. The development of acquired immunity is evidenced by the malarial crisis in which phenomenal numbers of parasites are eliminated by phagocytosis and their numbers in the blood are strikingly reduced. Acquired immunity appears to be an infection immunity, *i.e.*, it is maintained only by the continued presence of parasites somewhere in the body. With elimination of infection the individual soon regains susceptibility. Effective immunity appears to result from repeated infections and is best observed in adults in highly endemic areas. The enlarged spleen indicative of infection persists and, despite repeated reinoculation by mosquitoes, clinical malaria is seldom seen in persons long infected. The immunity is species specific in that immunity to one species of *Plasmodium* confers no protection against infection with another species. In addition, infection with one strain of a single species does not necessarily protect against immunologically different strains of the same species. Indeed, persons highly immune to *P. vivax* in one locality may be susceptible to strains from other areas.

Although immunity in malaria is basically dependent upon the removal of parasites by phagocytosis, humoral antibodies appear to play a role. Both complement-fixing and protective antibodies have been demonstrated experimentally. The only evidence of racial immunity in human malaria is the resistance of the Negro to infection with *P. vivax*.



**Control.** Efficient control of malaria depends upon a thorough understanding of the epidemiology of the disease, not only in general but in the specific locality. This information is obtained by surveys preceding the institution of control measures. The extent of infection is determined by examinations for parasites, enlargement of the spleen, and mosquito surveys are conducted to detect important malarial anophelines in the area and their breeding places. Although many species of anophelines may transmit malaria and several may be present in a given area, usually one species is found to be the important vector. In Europe *A. maculipennis*, in the United States *A. quadrimaculatus* and in Africa *A. gambiae* are the principal vectors.

Control measures consist of : (1) treatment of acute, chronic and latent infections with drugs in order to reduce sources of infection for mosquitoes as well as to benefit the individual; (2) destruction of adult mosquitoes or the use of protective measures against them; (3) destruction of mosquito larvae by chemical measures; and (4) destruction of mosquito breeding areas (see Chapter 1). In addition, suppressive drug treatment is often essential for newcomers to endemic areas, such as occurred during the recent war. Atabrine has proved a particularly valuable suppressive drug.

**Sarcosporidia** (Sarcocystis). *Sarcosporidia* are observed as crescent-shaped flattened parasites, 10 to 15  $\mu$  in length, within cylindrical tubes (Miescher's tubules) in striated muscle. *Sarcosporidia* have been found in many vertebrates including birds. Infection of man is rare and asymptomatic, the presence of the parasites usually being detected at autopsy. Studies of the mouse parasite have revealed that the spores are infectious by feeding. Following germination, the parasites penetrate the intestinal wall where multiplication takes place, and the young invade the smooth muscle fibers in various locations in the body and develop into the spore-containing Miescher's tubules.

**Toxoplasma.** Small, crescent-shaped or oval parasites, of the genus *Toxoplasma*, were described from rodents and are known to infect many species of these animals. The organisms are small, about 6  $\mu$  in length, and have a nucleus visible in Giemsa-stained smears. They are both extracellular and intracellular parasites, occurring either singly, in small clusters or as a mass of parasites (pseudocyst). *Toxoplasma* is present within phagocytic cells about air spaces within the lung in the pneumonic lesions of the typhus-like disease in adults and may be demonstrated in the brain in encephalitis in children. The strains appear to be identical immunologically and neutralizing antibodies develop during infection. Few cases of human toxoplasmosis have been recognized, but infection is likely more widespread than present information suggests. Encephalitis in children and infants is more frequently identified than is the disease in adults. The mode of transmission is unknown, but clinical evidence suggests *in utero* transference from the latently infected mother to the child as a method of dissemination.

**Other Sporozoa.** Numerous sporozoan parasites of lower animals, some of great economic importance, are recognized. *Babesia bigemina*, a small parasite

of the red blood cells, is important as the cause of Texas fever of cattle and the first parasite of animals shown to be transmitted by arthropods. In Theobald Smith and F. L. Kilbourne demonstrated transmission of *Babesia* ticks. Other blood sporozoa (*Haemosporidia*) cause malaria-like disease in birds. *Coccidia* are parasites of many vertebrates and other animals. The species which affects man, *Isospora hominis*, is a rare intestinal parasite, which may be a cause of diarrhea. The parasite is found in small numbers in the feces as small cysts or spores containing four sporozoites.

### MASTIGOPHORA

The flagellate protozoan parasites of man include systemic parasites of the blood and tissues of the family *Trypanosomidae* and parasites of the mouth, intestinal and female genital tracts. All are motile by means of one or more flagella at some stage in the life cycle. The *Trypanosomidae* are transmitted by biting insects, which become infected with a blood meal or, in some instances, directly from cutaneous lesions. The parasites of the body cavities are transferred from one host to another by personal contact or in water and food.

**The Trypanosomes and Leishmanias.** The trypanosomes and the leishmanias are important blood and tissue flagellates of man and the lower animals. Protozoa of the genera *Trypanosoma* and *Leishmania*, together with four genera of parasites of invertebrates (*Leptomonas*, *Herpetomonas*, *Crithidia* and *Phytomonas*) comprise the *Trypanosomidae*. These parasites are related by morphology and developmental cycle and all are flagellated in some stage of their life cycle. The trypanosomes are responsible for African trypanosomiasis (sleeping sickness) and the South American infection known as Chagas' disease in man, as well as for important infections of domestic and wild animals. The leishmanias are found in cutaneous infections (Oriental sore and mucocutaneous leishmaniasis) and the more serious systemic disease kala azar. Trypanosomes appear to have been recognized in animals as early as 1841. However, the flagellates were not associated with human disease until 1901 by Forde and later by Dutton, Castellani and Chagas. Leishmanias were found in kala azar in 1903 by Leishman and Donovan and in the same year were related to Oriental sore by Wright, although both parasites had been observed previously.

**Morphology and Cyclical Development.** **TRYPANOSOMA.** The trypanosomes are typically slender, fusiform or spindle-shaped protozoa with a single anterior flagellum. There is a single nucleus in the center of the cell and a posterior parabasal body and blepharoplast from which the flagellum arises. The flagellum is attached along the cell surface by a protoplasmic undulating membrane from its origin to the anterior end of the cell from which it extends forward (Fig. 180). Multiplication is by longitudinal fission and all stages of division and growth may be observed in preparations of fresh infected blood. The trypanosome form and, in some species, the leishmania form, which is morphologically identical to that of the genus *Leishmania* (see below), are found either in



food or tissues of the vertebrate host. The life cycle of the trypanosome characteristically includes residence in a blood-sucking insect, which serves as vector, as well as the parasitic stage in mammals. The parasites are acquired by the insect with the blood meal and undergo a cyclical development and multiplication within the alimentary tract of the insects which may include all morphological stages (leishmania, leptomonad, crithidia and trypanosome), after which infective parasites are eliminated either in the feces of the insect or from the salivary glands.

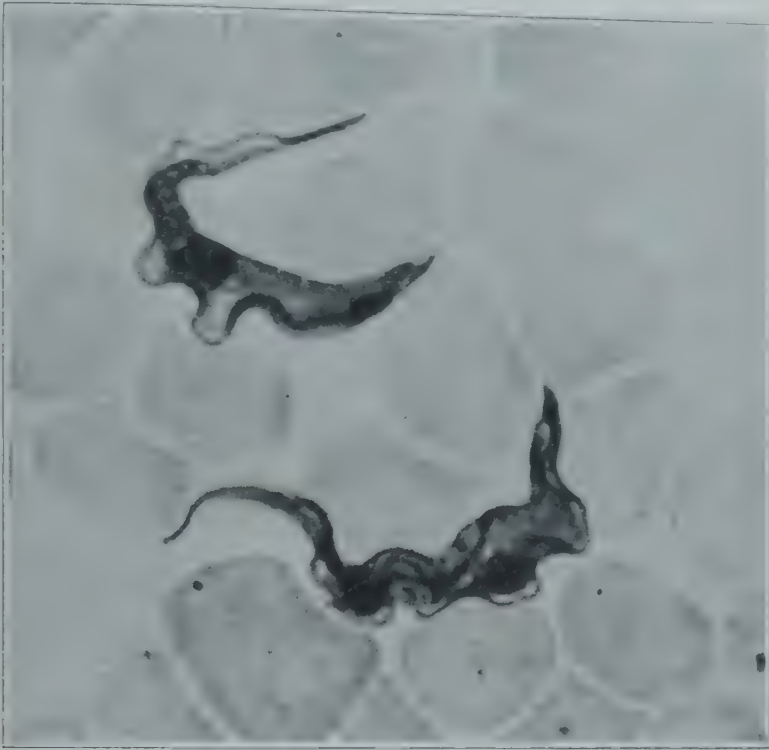


Fig. 180. Trypanosomes in a blood smear. Two trypanosomes are shown. Note the large, darkly staining nucleus, the smaller parabasal body from which the flagellum arises and the wavy undulating membrane along one margin of the cell. (Magnification approximately  $\times 1,600$ .) (*Trypanosoma equiperdum*, Kral.)

**LEISHMANIA.** The leishmanias exist in the human host as small (1 to 5  $\mu$  in diameter), round or oval, intracellular parasites, with a small nucleus and a kidney-shaped parabasal body and blepharoplast. In the insect host, however, both the amastigote leishmania and the flagellated leptomonad form are observed. The leptomonad form is also found in culture media, such as the N.N.N. (Novy-MacNeal-Noble) rabbit's blood agar.

**Immunity.** Untreated human trypanosomal infections are highly fatal diseases in which there is little evidence of immunity. These parasites are also highly pathogenic for laboratory animals, although some animals develop a relapsing disease, the relapses of which appear to be related to the production of successive broods of antigenically different parasites resistant to the immunity against their predecessors. Trypanocidal, agglutinating and complement-fixing antibodies may be demonstrated. Of particular interest is the demonstration of

specific reproduction-inhibiting antibody in trypanosomal infections of rats and mice. The specific antibody, **ablantin**, is present in immune serum. In leishmaniasis, on the other hand, recovery appears to be followed by solid immunity and second infections are rare.

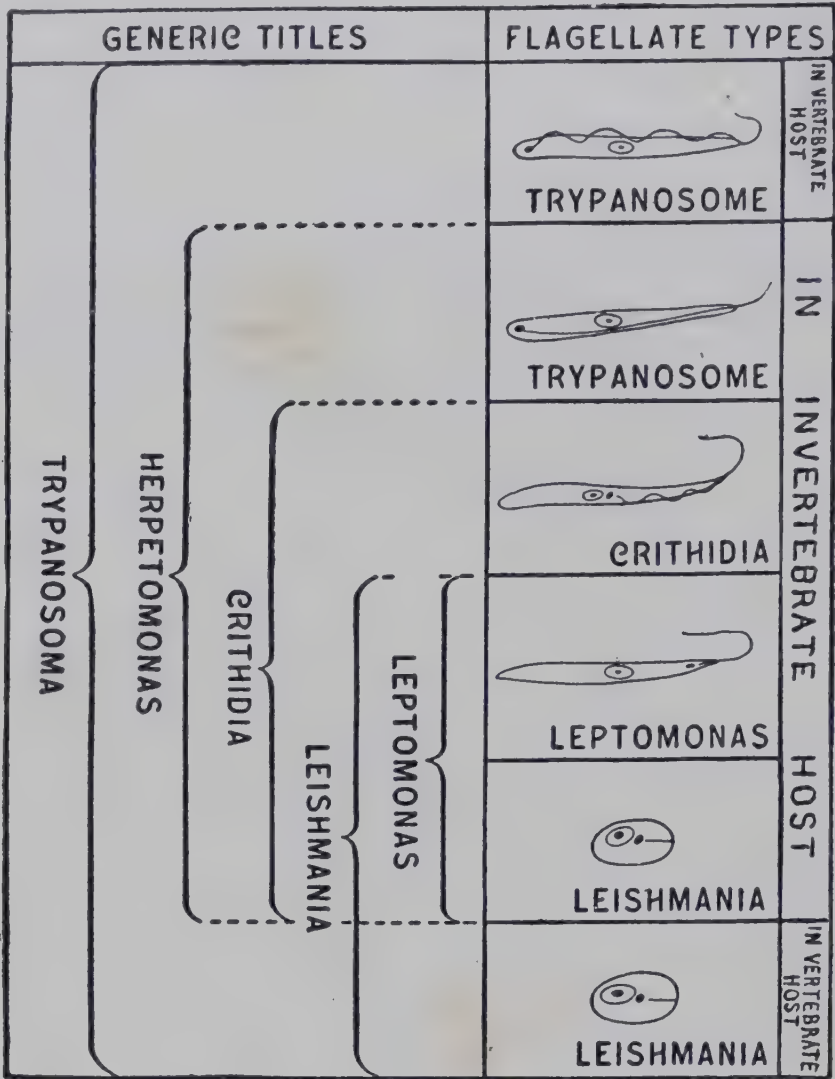


Fig. 181. Diagram of the stages in the life cycle of trypanosomidae. (After Wenyon from Hegner, Root, Augustine and Huff: *Parasitology*, D. Appleton-Century Co., Inc.)

**Trypanosoma Gambiense and Trypanosoma Rhodesiense** (African Sleeping Sickness). African trypanosomiasis or sleeping sickness is a febrile disease caused by *T. gambiense* and the closely related or identical *T. rhodesiense*. There are enlargement of the lymph nodes, inflammatory and destructive changes in the small blood vessels and involvement of the brain and meninges. The symptoms are those of a remitting fever with coma and death in the severe cases. The trypanosomes are found in the peripheral blood, the lymph nodes and the spinal fluid.

Two forms of the disease are recognized. The gambian type, caused by *T. gambiense* and transmitted by *Glossina palpalis*, occurs in tropical West and Central Africa. The more virulent Rhodesian type of East Africa is caused by *T. rhodesiense*.



and transmitted by *Glossina morsitans*. The disease does not occur outside the African continent and is limited in distribution by the range of the vector tsetse flies. Man is the principal host of *T. gambiense* and *T. rhodesiense*, although laboratory, domestic and wild animals may be infected. The antelope appears to be a natural animal reservoir of infection.

*Trypanosoma cruzi* (Chagas' Disease). South American trypanosomiasis (Chagas' disease) is limited in distribution to parts of Central and South America. *Trypanosoma cruzi* is found largely in the leishmania form in the cardiac muscle, liver, brain and other tissues, although flagellated forms may be found in the blood. Chagas' disease occurs in an acute form in children and as a chronic asymptomatic infection in older persons. The parasites are transmitted by blood-sucking reduviid bugs (*Triatoma*), particularly *T. megista*. Armadillos have been found infected with *T. cruzi* in nature and many laboratory animals are susceptible.

**Treatment and Control of Trypanosomiasis.** Trypanosomiasis may be treated by drugs of the arsenical series, particularly tryparsamide, in all stages of the disease and by the trypanocidal dye germanin (Bayer 205) in the early stages. Although these drugs are effective in African trypanosomiasis, the parasites rapidly become drug-fast, and the South American disease is unaffected by them.

Control measures consist of the treatment of known infections, the screening of premises to prevent access of insects, the avoidance of endemic areas or the use of protective clothing and measures directed against the insect vectors (see Chapter 45). Prophylactic chemotherapy has at times been recommended in endemic areas.

**Leishmaniasis.** Three species of *Leishmania* are parasites of man. *Leishmania donovani* is a systemic parasite responsible for kala-azar. *Leishmania tropica* and *Leishmania brasiliensis* produce cutaneous infections. These parasites are indistinguishable morphologically, but they differ in the types of disease which they produce. All are found within the macrophages in the leishmania form but produce flagellates (trypomastix) in culture and insect hosts. Several species of sandflies of the genus *Phlebotomus* have been incriminated as vectors of the leishmanias, although the evidence is in some instances incomplete. The flies abound in endemic areas, are known to feed on infected persons, and leptomonads may be found within the insects.

The leishmaniases yield to drug treatment with antimony compounds, most effective of which appear to be the pentavalent neostibosan and solustibosan and the aromatic diamide stilbamidine. In the cutaneous disease local x-irradiation or application of dry ice may be effective.

**Kala-Azar.** Kala-azar, systemic leishmaniasis, is prevalent in Asia, the Mediterranean region and Southeastern Europe. The incubation period, probably of several weeks' or months' duration, is followed by a febrile disease with splenic enlargement, emaciation, and changes in the blood. The Leishman-Donovan bodies (*L. donovani*) are found in abundance within the macrophages

of the spleen, liver and bone marrow. Indeed, cells of the reticulo-endothelial system are greatly increased in these locations and often show proliferation in other tissues. Diagnosis of kala-azar is made by the demonstration of *L. donovani* by microscopic examination or culture of the blood, aspirates of spleen or bone marrow and occasionally biopsy specimens. Serological tests to determine increases in euglobulin in the blood serum may be of value, but they become positive relatively late in the disease and reactions may be obtained in false conditions. In the formol gel (Napier's) test, 1.0 ml. of serum is mixed with one drop or two of formalin. Solid gel formation constitutes a positive reaction. In the antimony test of Chopra, a precipitate forms when diluted serum is mixed with a solution of urea stibamine.

**Oriental Sore.** Oriental sore or Delhi boil is a local, cutaneous, chronic inflammatory and ulcerating lesion which tends to spontaneous cure. Single or multiple lesions, which heal with scar formation, are usually found on exposed areas of skin. Cure is followed by permanent immunity against subsequent reinfection, an observation which in some areas has led to the practice of inoculation of children in unexposed areas of skin to prevent later infection and unsightly scarring. *Leishmania tropica* is demonstrable in smears of aspirates or curettage from deep portions of the lesions. Oriental sore is widely distributed in tropical and subtropical Asia, Europe and Africa. The infection is frequent in endemic areas and may assume epidemic proportions.

**Espundia.** Espundia, American or mucocutaneous leishmaniasis caused by *L. brasiliensis*, is a more serious infection than Oriental sore. Destructive ulcerating lesions containing *Leishmania* and leading to extensive scarring occur about the nose and mouth.

**Intestinal Flagellates.** The common intestinal flagellates of man are *Chilomastix mesnili*, *Giardia lamblia* and *Trichomonas hominis* (Fig. 17). These parasites are transmitted in contaminated food and drink. *Chilomastix* is a nonpathogenic, pear-shaped flagellate of the large intestine, which is transmitted in the cyst stage. *Giardia* is a binucleate, pear-shaped flagellate which has a cup-shaped ventral sucking disk. Resistant cysts contain four nuclei. *Giardia* inhabits the small intestine, particularly the duodenum, of approximately 10 per cent of the population, and it has been associated with diarrheal disease, although the majority of infections are asymptomatic. *Trichomonas hominis* is a parasite of the large intestine and may be the cause of diarrhea. Its pathogenicity, however, is not definitely established. Human trichomonads are not known to form cysts and are presumably transmitted from person to person as the trophozoite.

Trichomonads are also found in the mouth (*T. elongata*) and the female genital tract (*T. vaginalis*). The former is increased in numbers in gingivitis and pyorrhea alveolaris but is not known to be pathogenic. *Trichomonas vaginalis* is not clearly differentiated from *T. hominis* and may be the same species. Trichomoniasis is a common type of vaginitis, with inflammation, vaginal discharge and an altered bacterial flora. Diagnosis is made by demonstration of the motile parasites in fresh preparations of the vaginal secretion.



# Epidemiology and Public Health

## 43

### EPIDEMIOLOGY AND COMMUNITY DISEASE

Protection of the public health has contributed greatly to the development of modern civilization with its large urban communities. Indeed, the provision of safe water and milk supply, the proper disposal of sewage and many other control measures against the communicable diseases have been spectacularly successful and are essential to modern community living. These methods are based upon accurate knowledge of the relationships of hosts and parasites *en masse*, *i.e.*, epidemiology, and in essence consist of measures by which the balance between these two populations is shifted in favor of the hosts, particularly man. Epidemiology is concerned with the amount of disease and the factors which affect the behavior of disease in the community. Knowledge of the sources of infection, the portals of entry and exit and the means of transmission of infectious agents is, thus, essential to an understanding of epidemiology and to the application of control measures against infectious disease.

#### SOURCES OF INFECTION

Microorganisms pathogenic for man are, with few exceptions, parasites of man or the lower animals. They are seldom saprophytes, although many are capable of surviving for considerable periods of time on inanimate objects and in air, food and the soil. However, some parasites, such as the hookworms, undergo part of their life cycle in the soil and others, such as some of the flukes and the tapeworms, develop on plants and animals used for food. Sources of infection in the human population may be classified as typical cases of disease, atypical cases or inapparent infections. The typical case is highly infectious but is of danger to the community for a relatively short period of time. The isolation of the patient by illness and quarantine ordinarily reduces the risk of contagion by limiting his contacts. Atypical cases are often diagnosed with difficulty, or the disease may be so mild that the patient does not suspect the nature of his illness and does not seek medical advice. Active infections are, insofar as is known, the sole or principal sources of many infections, for example tuberculosis. On the other hand, many disease agents are spread by carriers of infection

who suffer no symptoms of disease but harbor and disseminate parasites among their associates. Patients recovering from a communicable disease, for example, often continue to harbor the causative agent for some time. The number of **convalescent carriers** is progressively reduced in the weeks and months following recovery so that only a few individuals remain chronically infected. **Chronic carriers** disseminate organisms continuously or intermittently for many months or years. Carriers of the intermittent type are particularly well known in the case of the enteric pathogens. Stool cultures from intermittent carriers of *Salmonella typhosa*, for example, may contain these organisms in large numbers at some times and be negative at others. On the other hand, continuous carriers emit organisms at all times. In contrast to the chronic carriers, susceptible individuals may be infected for only short periods of time. Such temporary carriers of respiratory tract pathogens, e.g., diphtheria, contribute to the spread of these agents of disease. Thus in diphtheria the carrier rate for an institution such as a school, may remain relatively constant, but the identity of the infected individuals may be quite different from day to day. The carrier may not have suffered an attack of disease at any time and may be recognized, if at all, only by the use of suitable culture methods. These subclinical, asymptomatic infections are well recognized in a number of diseases and are suspected in others. Carriers should be sought particularly among case contacts, institutional personnel, food handlers and others who have special opportunities to acquire and transmit organisms.

The lower animals, domestic and wild, are sources of infection for many of the many diseases of public health importance, as for example rabies, tularemia, brucellosis, salmonellosis, the rickettsial infections, bubonic plague, tapeworm infestations and trichinosis. Animals that harbor organisms pathogenic for man are termed **animal reservoirs of infection**.

### TRANSMISSION OF MICROORGANISMS

Transmission of infectious agents is essential for the continued occurrence of disease within the community. The development of new cases of disease is the evidence of the successful transmission of infection in the community. In general, the transfer of parasites may occur by direct contact, indirect methods and by living agents or **vectors**, such as insects.

**Transmission by Direct Contact.** Direct transmission occurs over short intervals of time and space through contact with fresh infectious material. Infectious tissues, human or animal exudates, such as respiratory droplets, direct personal contact, contaminated hands, inanimate objects and the air all may spread disease agents directly. The diseases transmitted principally by the direct route are often referred to as **contagious diseases**.

A variety of respiratory, skin and intestinal pathogens, including such important agents as those of scarlet fever, tuberculosis, typhoid fever and amebiasis may be transmitted from person to person by freshly contaminated hands or



mate objects. Similarly, personal contact is an important means of transmission of respiratory and skin pathogens, and it is the most important method of contracting the venereal diseases (see Chapter 45).

TABLE 20. OUTLINE OF MECHANISMS OF TRANSMISSION OF DISEASE TO MAN

TRANSMISSION BY DIRECT CONTACT.

- A. Diseases of man transmitted by direct personal contact.
  - Respiratory infections as diphtheria, scarlet fever, influenza and pneumonia.\*
  - Venereal diseases.
  - Intestinal infections as typhoid fever.
- B. Diseases of man transmitted by droplet and air-borne infection.
  - Respiratory infections as diphtheria, scarlet fever, and influenza.
  - Tuberculosis.
- C. Diseases of animals transmitted to man by contact.
  - Tularemia, psittacosis, foot and mouth disease.

TRANSMISSION BY THE INDIRECT ROUTE.

- A. Diseases transmitted by contaminated water.
  - Typhoid fever, paratyphoid fever, cholera, dysentery.
- B. Diseases transmitted by contaminated milk.
  - Typhoid and paratyphoid fevers, brucellosis, tuberculosis, scarlet fever, diphtheria.
- C. Diseases transmitted through contaminated food.
  - Typhoid and paratyphoid fevers, scarlet fever, *Salmonella* food poisoning, staphylococcal food poisoning, botulism.
- D. Diseases transmitted by fomites.
  - Many diseases such as typhoid fever and scarlet fever may be transmitted by contaminated books, towels, dishes, etc.

TRANSMISSION BY INSECT VECTORS.

Many diseases may be transmitted from man to man and from animals to man by insect vectors. (For detailed outline see later section.)

The diseases named are intended only as examples and do not constitute a complete list.

**Droplet and Air-borne Infection.** Droplet and the related air-borne infections account for the majority of the transmissions of pathogens of the respiratory tract. Droplets emitted from the mouth and nose during sneezing, coughing and, in smaller numbers, during talking heavily contaminate the air in the immediate vicinity of the individual. The larger droplets, which are the infectious particles in droplet infection, settle out of the air within a few feet of the source, but the smaller microscopic ones dry rapidly and remain suspended in the air. It has been shown that these buoyant, desiccated particles, called *droplet nuclei*, may in time be widely spread by natural air currents or ventilation systems. Bacterial droplet nuclei may be infectious for many hours, and influenza virus may remain suspended in air-borne droplets in infectious quantities for at least one-half hour. Thus, the air in the immediate vicinity of a patient or

carrier of infection will be heavily contaminated by both droplets and droplet nuclei, whereas at greater distances it will contain only the droplet nuclei. During periods of inactivity and vacancy the air of rooms tends to become purified by the settling of the particles. However, microorganisms that have settled onto the floor, furnishings or walls may be resuspended during occupancy and activities such as occurs during the performance of housekeeping duties.

**Transmission by Indirect Methods.** As the name implies, indirect transmission occurs through some medium, such as water, food, milk, soil and animate objects. In general, indirect transmission involves longer distances and longer times than does direct infection. Contaminated inanimate objects such as books, toys, towels, clothing and instruments (collectively known as **fomites**), water, food and milk are the vehicles of indirect transmission.

**Transmission by Insect Vectors.** A large and important group of diseases, including malaria, yellow fever, bubonic plague and typhus fever, are transmitted by living vectors, such as mosquitoes, ticks and mites. Parasites may be transmitted by the vector mechanically, as in the case of typhoid fever by house flies, or they may multiply and develop within the vector, as in the transmission of malaria by mosquitoes. The mechanisms of the transmission of disease by insects will be discussed in greater detail in a later section.

### THE AMOUNT OF DISEASE IN THE COMMUNITY

**Endemic, Epidemic, Pandemic.** Many of the common communicable diseases have occurred more or less continuously in our communities for many years, indeed for centuries; *i.e.*, they are **endemic**. The rate of occurrence of endemic disease tends to be relatively low and constant, but periodic outbreaks of greater severity or **epidemics** may occur. Other diseases are relatively restricted to one geographic region or to one group of the population and are significantly absent elsewhere. For example, Asiatic cholera is continuously present only in India and certain parts of China and, although it has occurred in epidemics in many regions of the world, the disease has not become established in the latter areas. Such localized geographical areas in which infection persists are known as **endemic zones** or **foci**.

A disease is said to be epidemic when an unusually large number of cases occurs within a short time. An epidemic may follow the introduction of a foreign disease into the community or, as indicated above, it may be caused by an endemic disease. For example, measles is endemic in most large communities and occurs in epidemics at intervals of about 2 to 5 years. This same disease causes severe epidemics when first introduced into some of the South Sea Islands. An outbreak of disease is usually referred to as an epidemic when it involves a limited geographical area, as for example one city or country. It may, however, spread through large geographical areas, such as one or several continents, in which case the outbreak is termed a **pandemic**. Influenza has repeatedly become pandemic, most recently in 1918 and 1919, when it invaded the major continents.



many islands before it finally subsided. Local epidemics of influenza are experienced at more frequent intervals.

**Rates.** The numbers of cases and deaths from disease are conveniently expressed by rates. **Mortality** or **death rates** are usually calculated as the number of deaths per 1,000 or 100,000 population. The **morbidity** indicates the number of cases of illness per population unit, usually per 1,000 persons. In other words, rates of importance to the public health express the number of times an event (disease, death, birth, etc.) occurs within a certain group of the population. If the total population, irrespective of race, sex or age, is used in the calculation, the rate is referred to as a **crude rate**. Particular groups of the population are, however, affected by certain diseases to a greater extent than are other groups, so that **specific rates** are of great value. For example, age-specific death rates indicate trends in diphtheria more clearly than do crude rates, since this disease is more prevalent among children than among adults. Although specific rates most often express the number of times an event occurs in particular race, age and sex groups, they may refer to any limited group of the population.

**Sources of Data.** The basic information for disease and death rates is contained in the reports of births, deaths and cases of disease made to local health authorities. Births and deaths must be recorded on legal certificates. Likewise, cases of certain diseases, infectious and otherwise, must be reported to local health authorities. The laws governing these disease and death reports are different laws and differ among the several states. The requirement of registration of births and deaths is, however, common to all states. The data of the census also furnish much information, such as the number, age, sex, race and habitation of the people. In addition to the above, disease statistics are sometimes obtained from surveys of limited groups, such as the armed forces, industrial employees or residents of a particular city.

The importance of completely reporting diseases should be emphasized. Without accurate information the health department can perform only with difficulty its function of protecting the public health.

**Factors Affecting the Amount of Community Disease.** The amount and severity of disease within the community is probably best looked upon as the result of the number of individual cases and is, therefore, affected by the virulence and number of the infecting organisms, the susceptibility of the hosts and the transmission of the parasites. As previously indicated, variation in virulence of a single strain of microorganisms under natural conditions seems to occur at a very low rate and is seldom observed during a single epidemic. On the other hand, different strains differ in virulence, so that the severity of clinical symptoms may be related to infection with benign or malignant varieties of organisms. Epidemics of benign and malignant smallpox have been directly related to the virulence of the causative virus, and recently severe and benign diphtheria has in some instances been attributed to infection with *gravis* and *mitis* strains of the diphtheria bacillus. The epidemiological picture is further complicated by the presence of strains of both high and low virulence in the same epidemic. During the rise of an

epidemic, however, cases progressively increase in number and severity, a phenomenon which may be explained to a large extent by the increasing exposure of susceptible individuals during this period. The dosage may become sufficiently great to infect a number of partially immune individuals resistant to small numbers of organisms. In the long view changes in virulence of the causative organisms are important to the character of infectious disease and undoubtedly account in part for the changes in infectivity and severity of disease observed over long periods of time.

Susceptibility of the population is an extremely important and frequently determining factor in the causation of epidemics. The residents of isolated, disease-free communities are almost uniformly susceptible and may be severely affected by newly introduced infection. On the other hand, many individuals in endemic areas are immune, so that communicable diseases may attack chiefly the relatively new and previously unexposed residents. For example, the common contagious diseases scarlet fever and measles occur largely among children, although nonimmune adults may succumb to infection. Susceptible individuals are recruited through births and immigration. An epidemic may occur only if the proportion of susceptibles is sufficiently great to permit effective transmission of the disease agent from infected to susceptible individuals. If the population is highly immune the infection of susceptibles becomes relatively unlikely and the disease either occurs at an endemic rate or may die out locally. During the course of an epidemic the proportion of susceptibles is reduced and, consequently, the number of cases thereafter declines; as the fuel is consumed the fire burns low. This decline frequently can be hastened or the epidemic may be aborted by the widespread use of artificial immunization, the prevention of contact by isolation of patients and by sanitary measures. Under natural conditions the concentration of susceptibles is slowly rebuilt following one epidemic and when sufficiently great may support a new epidemic. This cycle of epidemic rise and fall and its recurrence may, as in the case of measles, be repeated at predictable intervals or it may occur irregularly.

The mechanisms of transmission most likely to be related to epidemics are those capable of conveying organisms rapidly to many individuals. Infectious droplets, the air, food, milk and insect vectors are, therefore, likely to be implicated in outbreaks of disease.

**Social Factors and Communicable Disease.** The number and severity of cases of communicable disease are affected by many socio-economic factors. Poverty, crowding, malnutrition, methods of transportation and occupation are important. The expansion of modern economy to include the entire world has created an interdependent community and the development of modern transportation have broadened and accentuated the effect of these factors on the occurrence of disease.

It is axiomatic that the spread of disease follows the routes of human travel. Whereas formerly the speed of transportation was limited to that of the caravan and sailing ship, today it is determined by the train, automobile, steamship and



plane. In the absence of control measures parasites may be transported around the world in a few hours by airplane, and by other means may cross continents and oceans in a few days. Disease agents may be spread by these routes in several ways. Infected human beings may be transported and, by air, arrive at their destination while in the incubation period of many diseases. Infected animals, particularly rodents such as rats, may be carried aboard ships, planes and other craft. Insect vectors, as for example the mosquito and the body louse, may be conveyed separately or on their hosts.

Protection against the transportation of disease agents by these methods presents many problems and requires constant vigilance. Control measures that have proved effective include inspection of craft and passengers by trained personnel at ports of departure and arrival, quarantine, disinfection and fumigation. These methods have been long enforced for sea traffic and with technical modifications are being applied to transportation by air.

Crowding increases the incidence of disease by increasing individual exposure. The effects of crowding are particularly severe during war and natural catastrophes when people from wide geographical areas may be housed in common shelters or brought together in army, refugee and other camps. Close association increases the transference of organisms by droplets, the air and body parasites, such as the louse. Infection is further aided by disruption of water supplies and sanitary facilities. During such catastrophes the incidence and mortality of influenza, pneumonia, diphtheria, typhoid fever, tuberculosis, typhus and other diseases are commonly greatly increased. Under normal circumstances the effect of crowding is less obvious but nevertheless present. People are closely associated in homes, schools, dormitories, theaters, factories and meeting places of various types. Catastrophes and congregation cannot be eliminated, but improvements in housing, sanitation, including control of air-borne disease, and working conditions are being made. During epidemics, attendance at schools, theatres and meetings may be temporarily interrupted in order to reduce the number of contacts.

Epidemics of communicable disease frequently follow famine. Malnutrition, particularly protein deficiency, lowers the capacity of the individual to resist infection. Malnutrition, like crowding, is widespread during war, but it is by its means limited to this or other catastrophes.

High incidence of certain diseases is correlated with occupation. Thus an unusually high frequency of tuberculosis has long been recognized among miners, stone cutters and workers with abrasives. The lungs of these workers are a particularly fertile field for development of this disease because of serious injury by silica dust. Workers with unsterilized wool and hides are particularly exposed to the risk of anthrax. Other instances of occupational relationships of the infectious diseases have been cited in sections dealing with these diseases. Although important, special occupational risks are limited to relatively small groups of the working population. Of far more concern are the loss of time and income from diseases that affect the general population, and

which have a high incidence among workers, industrial or office, because of close contact rather than occupational hazard. Thus the acute respiratory infections are the first cause of illness absenteeism in various industries, and tuberculosis, aside from the instances of special risk cited above, presents much the same problems in industry as in the general population.

Although industry, because of the association of large numbers of people, may suffer unduly from infectious disease, it affords unusually good opportunities for mass control of many diseases. For example, case detection and treatment of tuberculosis in industry can benefit the worker, his family and the community. The same is true of the venereal diseases. Prevention of infection by treatment of the air is in its infancy, but this too appears well adapted to use in plants and office. Industrial public health thus plays a role in the prevention and control of many infectious diseases, general as well as occupational.



## THE COMMUNICABLE DISEASES

### ACUTE RESPIRATORY DISEASES

The respiratory diseases include the common cold, influenza, virus and bacterial pneumonia, measles, mumps, pertussis, chickenpox, smallpox, meningococcal meningitis, scarlet fever, diphtheria and, at least in some instances, poliomyelitis. The chief mode of transmission of these diseases is by air-borne droplets and droplet nuclei. They may be transferred also by contaminated hands, instruments, clothing, and bedding, and, less frequently, by milk and food. Milk-borne epidemics of streptococcal infection and diphtheria are well known. Is food-borne streptococcal disease.

The respiratory diseases are frequent in temperate climates, but the tropics do not escape their virulence. Measles, diphtheria, influenza and pneumonia are reported from all climates. In temperate zones the prevalence of all these diseases, with the exception of poliomyelitis, increases sharply in the fall of the year, and continues to be high throughout the cold months. Major epidemics may be encountered at any time during this period, but often the peak incidence does not occur until Spring. They are less prevalent in the Summer. The seasonal incidence of some of these diseases is shown graphically in Figure 182. They tend to spread rapidly through asylums, military barracks, schools and dormitories. Although many of these diseases have in recent years become less frequent, epidemics still occur every few years. Others, such as the common cold and measles, continue to have a high incidence. The most important factor determining the seasonal incidence is the opportunity for infection afforded by indoor crowding during the cold months of the year, although chilling, poor nutrition and other nonspecific factors undoubtedly play a role.

The high morbidity of diseases, such as measles, scarlet fever, and diphtheria, among children is related to their lack of previous exposure, for adults who have hitherto escaped infection often succumb.

Control measures include isolation and treatment of patients and carriers, widespread use of prophylactic immunizing agents such as diphtheria toxoid, and the disinfection of air. The isolation technique employed against these diseases is often referred to as respiratory isolation. It includes isolation of patients to avoid cross infection, the use of cap, mask and gown by all who come into contact with the patient and the thorough disinfection or heat sterilization of all articles, including instruments, used in the isolation unit.

Hands must be carefully disinfected and washed by everyone leaving the isolation. Unnecessary exposures are to be avoided. Education of the public concerning isolation, the dangers of exposure, and the reporting of cases plays its important role.

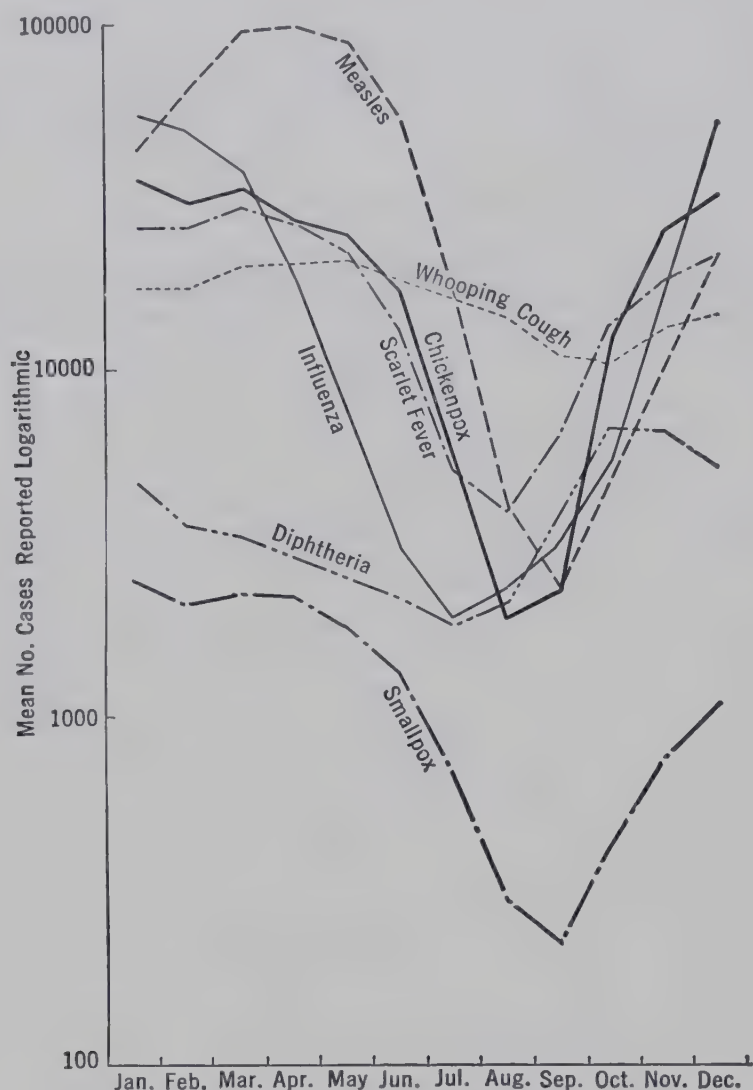


Fig. 182. The seasonal incidence of some acute communicable diseases. (Data from Supplements to the *Public Health Reports*. Courtesy of Dr. Wm. Burrows.)

Disinfection of the air has recently been successfully accomplished in experimental tests, and will probably be widely used in the future. Air disinfectants or germicidal aerosols, as they are called, have the property of being bactericidal when suspended in the air in very low concentrations, provided the air contains a reasonable amount of moisture. The aerosol is believed to become concentrated and to be germicidal within the tiny droplets containing microorganisms. Of the many agents that have been tried, propylene glycol and tri-ethylene glycol concentrations of one part in several million parts of air are among the most promising in experimental tests. It is probable that these agents will be widely used in the control of air-borne infection. Ultraviolet light also is applicable to air disinfection and may be used at the entrance to isolation cubicles and



er room air. Persons may be directly exposed to the rays only for short periods of time, however, without suffering deleterious effects. Dust control and general good housekeeping likewise are important in the control of air-borne infection.

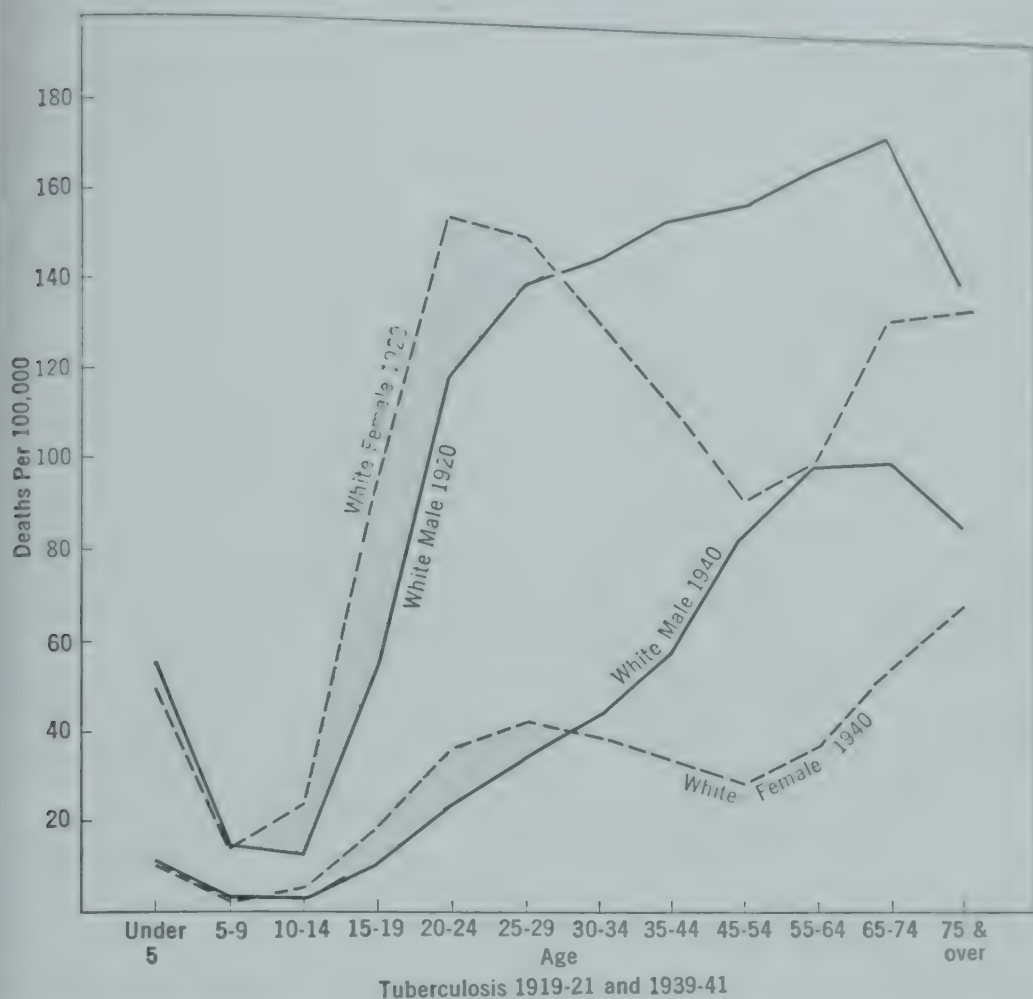


Fig. 183. Age and sex distribution of mortality from tuberculosis in the white population. Note reduction in 1940 compared to 1920. (Data from Yerushalmy, Liboe and Palmer: *Public Health Reports* 58: 1457, 1943.)

## TUBERCULOSIS

Despite the great improvement in mortality during the past century, tuberculosis continues to be one of the major public health problems. The death rate in this disease has been reduced to a far greater extent than has the incidence of infection.

Tuberculosis may be acquired from either infected human beings or cattle. The most infectious material is tuberculous human sputum. In this medium the organisms may be spread by direct contact and air-borne droplets, and the bacteria in dried sputum may be dispersed in the air with dust. At the present time approximately 90 per cent of tuberculosis primarily involves the respiratory tract. Second in importance as a source of human disease are tuberculous cows. Milk-borne tuberculosis, however, has been greatly reduced by the elimination of positive tuberculin-reacting cows from dairy herds and by milk pasteurization.

Tuberculosis is a disease of contact, of civilization and of cities. It is widespread in barracks, jails, asylums and other closed communities and is particularly prevalent among family contacts of cases. On the other hand, nomadic and isolated peoples are remarkably free of infection, although

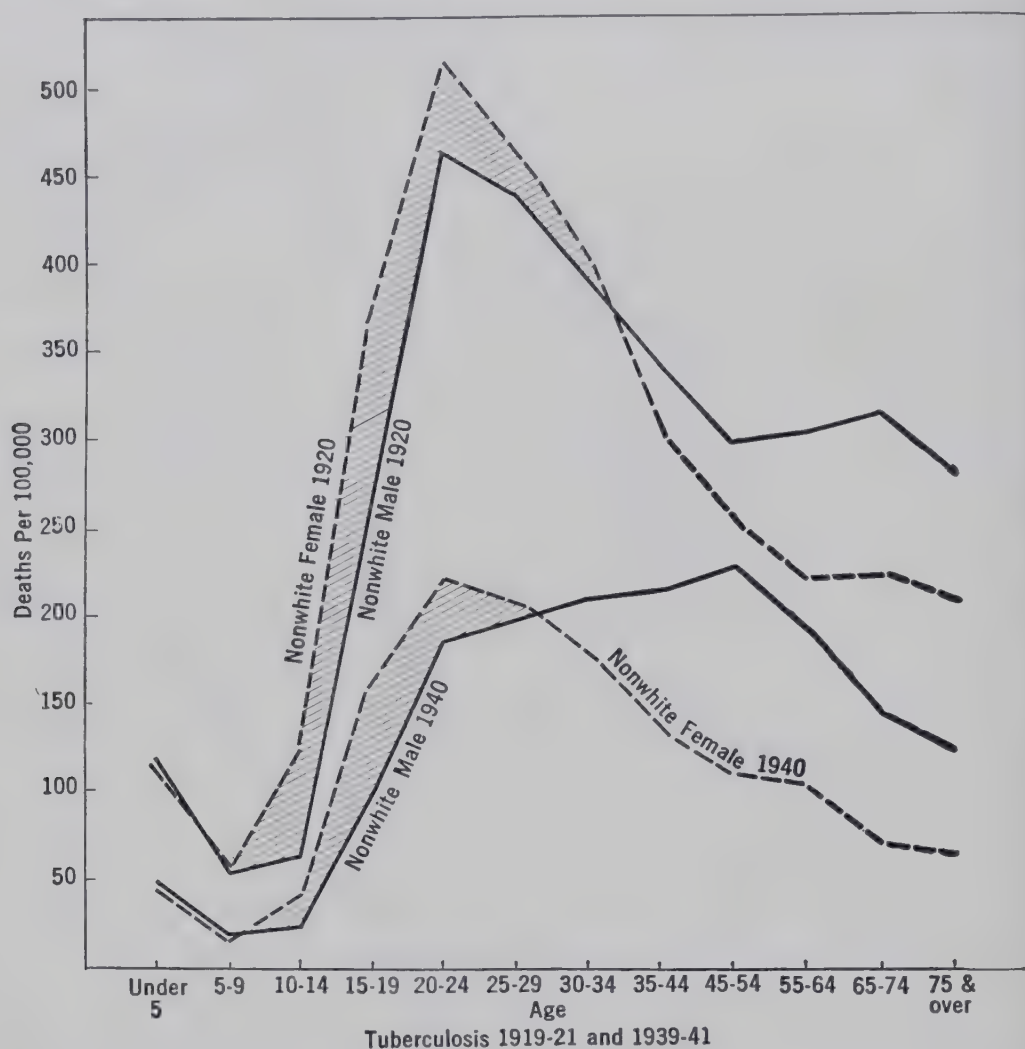


Fig. 184. Age and sex distribution of mortality from tuberculosis in the nonwhite population. Note the reduction in mortality between 1920 and 1940 and the high mortality as compared to that of the white population. (Data from Yerushalmy, Hillier and Palmer: *Public Health Reports* 58: 1457, 1943.)

same people when exposed prove exceptionally susceptible to the disease and succumb in large numbers.

Although the mortality from tuberculosis is only about one-fourth that at the beginning of the century, the disease remains one of the first three causes of death between the ages of 15 and 49 years, and is responsible for about 20 per cent of deaths in the 20- to 30-year age group. It affects Negroes much more severely than white men. At the beginning of the century the mortality was high among infants and young children, but it has been greatly reduced in the last forty years through detection and treatment of tuberculosis in prospective mothers and the supervision of the children of tuberculous parents from birth. Important also have been the pasteurization of milk, the removal of patients from the home and the care of child contacts in preventoria.



Today case detection is one of the most important public health problems in tuberculosis. The disease is insidious, and so-called early symptoms appear relatively late in its course. During the symptom-free early period the patient is likely to be a source of infection to others, and without treatment his own disease progresses. Until recently diagnosis was most often postponed until symptoms brought the patient to the physician. Although this is still true in many instances, development of techniques for the x-ray examination of large numbers of people promises to alter the situation in the future. The miniature x-ray technique, introduced a short time before the recent war, is particularly well suited for this purpose. It is inexpensive, convenient and gives satisfactory results. This technique has been used extensively by the armed forces and is being extended to industrial and other groups. In the future the x-ray examination for tuberculosis promises to be as widely used as the serological test for syphilis.

The tuberculin test is also of value in detecting tuberculosis. However, its interpretation is sometimes difficult, particularly in adults, since 50 to 75 per cent of the population may be positive reactors by the age of 15 years. Nevertheless, tuberculin reactions are significant in children and in adults who, formerly negative, have become positive. Alone the tuberculin reaction must not be interpreted as meaning active disease, but in surveys it is a useful adjunct to other methods of examination. The tuberculin reaction is assuming added importance in the survey diagnosis of tuberculosis because of the demonstration that histoplasmosis and coccidioidomycosis, fungus diseases, are important causes of chronic pulmonary disease.

In the hospital, the isolation technique described for the respiratory diseases must be enforced. All instruments, trays, utensils and other equipment must be disinfected or steam-sterilized. Two per cent cresol solution is a satisfactory disinfectant.

In the community every effort should be made to isolate patients in sanatoria properly staffed and equipped to give the special care required for these patients. Case detection surveys should be made periodically. These can probably best be carried out in industry, schools and in prenatal, child welfare and general clinics. Furthermore, an attempt must be made to detect early disease in contacts of known patients, particularly in members of the family. Education is of the first order of importance in the control of tuberculosis. Recently experimental trials with B.C.G. (Bacille Calmette-Guérin) vaccination of children both in this country and abroad strongly suggest the value of this procedure in the prevention of childhood tuberculosis. However, the trials are as yet inadequate to determine the value and safety of this procedure in general public health practice.

## GASTRO-INTESTINAL INFECTIONS

The major diseases of the enteric group are typhoid and paratyphoid fevers, shigellosis, Asiatic cholera, bacillary and amebic dysentery, the bacterial food poisonings, infections with higher animal parasites and, possibly, poliomyelitis. The causative organisms of these diseases enter the body through the gastro-

intestinal tract and leave it in the feces or occasionally in the urine. Spores of these organisms may be transmitted by one or several of the following ways: contaminated water, food, milk, hands and inanimate objects (fomites) and domestic flies. Spread of the enteric pathogens by flies is secondary but nevertheless important. These insects transmit the organisms mechanically.

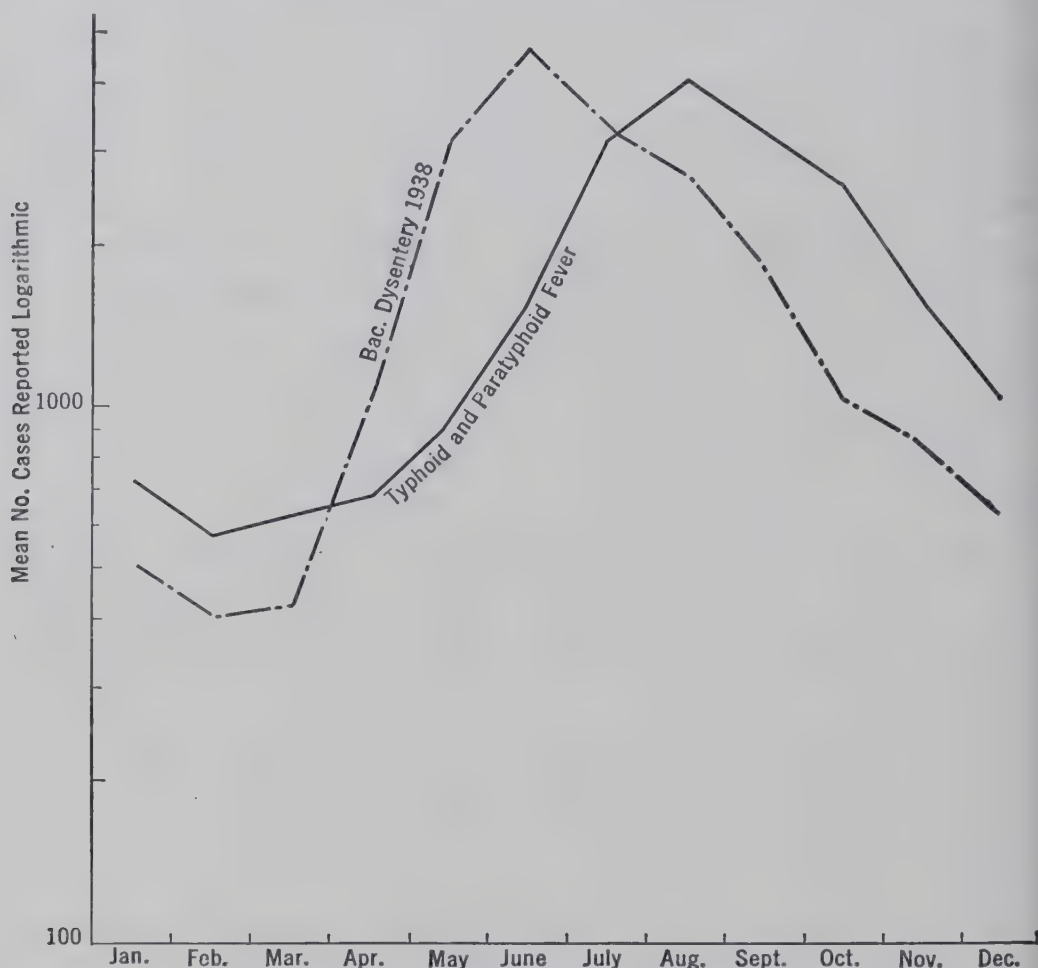


Fig. 185. Seasonal incidence of bacillary dysentery and typhoid fever. (Data from Supplements to the *Public Health Reports*. Courtesy of Dr. Wm. Burrows.)

Patients and, in many diseases, carriers and animal reservoirs are the source of infection. Indeed, epidemics can frequently be traced to a single case or carrier. Typhoid carriers, for example, have been found among food dispensers and dairy workers, all of whom have ample opportunity to disseminate organisms. In other instances, sewage from the residence of a case or carrier has contaminated the water supply of an entire city, resulting in an epidemic of the disease.

Control measures against these infections consist in isolation and treatment of patients, detection and treatment of carriers, disinfection of utensils used in the sickroom, sanitation of water, food and milk and protection against flies. Protective immunization is necessary only in the event of unusual exposure, such as occurs during vacations in rural areas, war and natural catastrophes. The isolation technique differs in certain respects from that described for the respiratory diseases. The mask, so important in respiratory isolation, may often be omitted.



The cap and gown should be worn by all caring for the patient. The room must be adequately screened against flies. Disinfection of hands, instruments and linens is important, as is the sterilization of linens, dishes and bedpans. Cresol (1) or other disinfectant must be added to wastes and excrement before disposal. Food and milk handlers should submit to periodic medical and laboratory examination to ensure absence of pathogenic organisms. Persons in the infectious stage of active disease or those having inapparent infections should not be employed by establishments dealing in foods. Known carriers are subject to supervision by the health department. They should be given available treatment, instructed in methods of personal hygiene and, if necessary, isolated. Sanitation of food and milk will be discussed in the following sections.

**Water and Disease.** Provision of an abundant and safe water supply has become a recognized function of government except in those regions, chiefly rural, where the water supply is still a family project. Even in these areas samples of water may be submitted for analysis to laboratories, and a report on the safety of the supply obtained within a short time. Such, however, has not always been the case, and contaminated water has been and, without proper precaution, in rural communities may be responsible for disastrous epidemics.

**Contaminated water** may be defined as water that contains human or animal waste products or poisonous chemical substances. The former is by far the most important form of contamination from the public health point of view. Contaminated water may by inspection seem suitable for drinking. If its physical properties are impaired the water is said to be polluted. The sanitary examination of a water supply is designed to detect the existence and source of contamination and pollution. A complete analysis includes: (1) a field survey for detection of sources of contamination, (2) physical and chemical analysis, (3) microscopic and bacteriological examinations.

The total number of the bacteria in water is roughly proportional to the degree of pollution. Since human and animal waste products are the major sources of disease agents in water, the presence and number of fecal organisms are of primary concern. The fecal organisms, specifically members of the *coliform* group, are used as an index of fecal contamination because they are present in large numbers in feces, are easily identified and have approximately the same resistance to deleterious agents as do the pathogens. Pathogenic organisms, such as *Salmonella typhosa*, are not sought because they are present in relatively small numbers even in grossly contaminated water. Furthermore, water that contains fecal organisms may also contain pathogens and is potentially, if not actually, infectious.

**The Bacteriological Examination of Water.** The bacteriological examination of water consists of: (1) the plate count of viable bacteria and (2) the determination of the coliform group of bacteria. The actual procedures for these and other tests, chemical, physical and microscopic, for the examination of water are specified in *Standard Methods for the Examination of Water and Wastewater*, published by the American Public Health Association. Samples of water for the tests are carefully collected in clean, sterile bottles, which are then

stoppered and rapidly transported to the laboratory. The total number of bacteria is determined by counting the number of colonies which develop on nutrient agar pour plates inoculated with appropriate dilutions of the water sample. The plates may be incubated at either 20° or 35° C. It should be remembered that, whereas coliform organisms grow at both temperatures, bacteria develop better at 20° C. The presence of coliform organisms is determined by: (1) observation of formation of gas in lactose broth fermentation tubes at 35° C within 24 hours (the **presumptive test**); (2) the isolation of typical colonies of *Bact. coli* on Endo's or eosin-methylene-blue medium pour plates (the **confirmed test**); and (3) the observation of formation of gas in lactose broth fermentation tubes inoculated with organisms from a single colony (the **completed test**).

Certain alternative procedures are permissible. Graduated portions of a water sample, usually totalling 50 or 100 ml., are examined for coliform organisms. The occasional, but not the continued, presence of coliform organisms is permitted in water suitable for drinking. In the event of contamination (*i.e.*, presence of coliform bacteria) repeated tests should be performed and appropriate protective measures instituted. The number and frequency of routine examinations are based largely upon the size of the supply and the previous reports of results of examinations. Periodic examination of all water supplies should be carried out in order to ensure purity of the supply. Occasionally, differentiation of members of the *coli-aerogenes* group is indicated, but this is not a routine procedure.

**Sources of Water Supply.** Water for a community supply may be obtained from surface water, such as lakes, rivers and reservoirs, and from ground water, wells and springs. Unfortunately the most abundant of these, surface water, is also usually contaminated. Ground water is less likely to be contaminated, but it is available in quantities sufficient only for small communities or individual dwellings. Since water from either source may contain harmful microorganisms, both should be supervised. Contamination of an urban water supply most often occurs at its source, but it may result from faulty conduits or an error in pipe connections, so that occasionally the water of a single building may be contaminated while that of other parts of the city remains pure. Wells may be contaminated either at the surface through leaking platforms and casements, or beneath the surface from a nearby faulty sewer, cesspool or septic tank. If the well is situated in limestone, impure water flowing in fissures in the rock may contaminate the supply.

The wide distribution of an urban water supply favors the spread of pathogenic organisms to a large proportion of the population at one time. Hence outbreaks of water-borne disease tend to begin suddenly or explosively. The number of cases of disease depends upon the amount of contamination and the distribution of the supply. If both are great, the epidemic will probably be severe. In other instances only a small number of cases may occur. In either instance an epidemiological survey will reveal a close correlation between illness and



use of the contaminated water. Water-borne disease affects both sexes and races and age groups. Furthermore, epidemics traceable to contaminated water have occurred in all seasons of the year.

**Water Purification.** Public health textbooks should be consulted for a discussion of methods of water purification, since only a few methods may be added here. In an emergency, small quantities of water may be purified by boiling for 10 to 15 minutes. Chemical purification, particularly chlorination, may be used either as an emergency measure or for purification of large urban supplies. Municipal supplies are often purified by compressed gaseous chlorine, which is added to the water in amounts of 0.25 to 2.0 parts per million of water, depending upon the degree of pollution and the nature of the supply. Chlorine in adequate amounts supplies a residual concentration which protects the water supply throughout the distribution system. Chlorinated lime may be used to purify either municipal or small supplies. This substance is added to water in amounts proportional to its available chlorine content (about 35 per cent). One advantage of chlorinated lime is that it is an unstable compound and loses potency during storage. Chloramines (compounds of ammonia and chlorine) are also widely used for water purification, and recently certain organic chlorine compounds, such as halazone (NNR), have been suggested for emergency use on a small scale.

Water may also be purified by filtration. It is passed through graded sand, usually after preliminary sedimentation to remove a large part of the suspended material, and is then collected in mains beneath the filter bed. The water collected from properly constructed and operated filters is safe for drinking and physically improved, but without further treatment may be contaminated thereafter. Filtration of ground water is usually unnecessary. Combined filtration and chlorination is the most satisfactory and adequate method of protecting water obtained from surface supplies.

Formerly reliance was frequently placed upon the purification of water by sedimentation of bacteria and their destruction by sunlight during storage in impounded reservoirs. This method of natural purification has been largely abandoned, however, as an uncertain means of protection. Impounding of water is still of value but it should be supplemented by more reliable methods of purification.

**Sewage Disposal.** Sewage consists of a mixture of human, animal, household, industrial and street wastes and water. Since it is the chief source of pollution and contamination of water, the methods of its disposal assume great public health importance. These methods are concerned with the disposal of large quantities of waste products in a manner that minimizes stream pollution and the danger of contamination of a water supply.

Sewage disposal by dilution, *i.e.*, directly into bodies of water, is usually unsatisfactory because of the degree of pollution of the water. Treatment methods are therefore commonly employed. Sewage treatment consists of **preliminary processes**, including screening to remove coarse particles and sedimentation for

removal of sand, grit, other settling solids and oils, **key processes** of bacterial digestion and oxidation of the contained organic matter, and finishing processes, as secondary sedimentation, chlorination and disposal of the treated sewage. The liquid effluent remaining after treatment may be disposed of by dilution in streams and the solid residue, *i.e.*, the sewage sludge, after further treatment is incinerated, sold as fertilizer or dumped into lowlands or streams.

Sewage treatment is in essence a biological process of decomposition and oxidation of complex organic substances, such as carbohydrates and proteins into simpler inorganic compounds. In streams or the soil the process is known as **self-purification**. Whether the result of natural processes or artificial treatment, the changes are brought about by the activity of microorganisms and may result from either aerobic or anaerobic decomposition. Aerobic decomposition is a more efficient process and results in a more completely digested and sanitized effluent which may be readily disposed of without the creation of a nuisance.

Although specific measures for their destruction are not a part of the normal processes of sewage treatment, fecal bacteria, including enteric pathogens, are rapidly reduced in number and it is unlikely that they survive the better treatment methods. Final disinfection of the effluent is, however, necessary to insure the destruction of enteric bacteria and is required for the protection of shellfish beds. Chlorine is the most commonly used agent. Ordinarily reliance is placed upon water purification methods rather than upon sewage disposal to insure the absence of contamination of drinking water. Hospital wastes should always be disinfected before disposal.

The disposal problems of rural communities, camps and family groups are chiefly those of the sanitary disposal of fecal material and kitchen wastes. Privies, latrines, cesspools and septic tanks must be so constructed and so located that they do not endanger the water supply or permit access by flies. Bacterial decomposition and oxidation are the key processes here as in the other disposal methods. Final disposal may be by incineration or burial of the accumulated solid material not digested by the microorganisms.

**Milk and Disease.** Milk is our most important food, both from the standpoint of nutrition and the transmission of disease. Supervision of the milk supply is, therefore, of primary importance and it must guard against contamination and adulteration of fresh and processed dairy products.

Most bacteria grow readily in milk, so that a small initial number may increase to many millions per milliliter before the milk is consumed. Fresh milk is not sterile even when obtained from healthy cows, and additional organisms are introduced from the skin of the animal, dust, the air, utensils and the milk handler. Fortunately, most of these bacteria are harmless, although they are responsible for spoilage. Milk may be contaminated with pathogenic organisms either from human or bovine sources. Milk handlers may contaminate the milk directly or, more rarely, they may infect the cow, who then eliminates the organisms in her milk. Cows suffering from a number of bovine diseases may be the source of bacteria pathogenic for man in the milk, as for example bovine



ple bacilli. The diseases that may be transmitted in milk are tuberculosis, typhoid and paratyphoid fevers, brucellosis, bacillary dysentery, streptococcal infections, diphtheria, foot and mouth disease, staphylococcal food poisoning and possibly poliomyelitis.

Milk-borne epidemics vary in severity from a few to many cases depending on the degree of contamination and the distribution of the supply. The origin of such epidemics can usually be traced to a single dairy, and often to one diseased cow or milk handler. Epidemics tend to appear suddenly and rise to a peak rapidly. They may decline more slowly; new cases due to secondary infections and to the long incubation period of some diseases continue to be reported for some time after the milk has been adequately protected. Milk-borne epidemics may occur at any season of the year, but they are somewhat more frequent during the summer months.

Public health protection of the milk begins with supervision of the farm and ends with delivery to the customer. Not only whole milk but also cream, butter, cheese, ice cream and processed milk foods should be supervised. Except for interstate carriers the laws governing milk production are local. However, there is a tendency for cities to adopt the standard milk ordinance recommended by the U. S. Public Health Service.

Complete protection of milk cannot be assured without adequate pasteurization. A recommended technique consists in heating the milk to 142–145° F for 30 minutes. The flash method of pasteurization in which the milk is heated to a higher temperature for a shorter time period is sometimes employed. The pasteurization processes must be carefully supervised in order to ensure their efficacy.

Although pasteurization is a cheap method of ensuring safe milk, it is sometimes improperly carried out and is not a substitute for cleanliness in production, from herds of disease-free cattle. Dairy animals should be examined at intervals by competent veterinarians and should be free of tuberculosis and brucellosis. Milk handlers, likewise, should be healthy and should not be carriers of disease agents transmissible in milk. Milk should be stored in sterilized containers and properly cooled from the time of production until it is consumed.

The sanitary quality of milk may be determined by a number of methods, such as the bacterial plate count, the Breed count and the reductase test. The bacterial plate count is a measure of the viable bacteria in milk and, for this purpose, is perhaps the best index of quality. Indeed, milk standards grade milk partly by the number of viable bacteria present. Both the number of bacteria and the pus cells or leucocytes may be estimated by direct microscopic counts, such as that devised by Breed. The methylene blue reduction test (reductase test) provides a rough estimate of the number of bacteria in milk. It consists in the observation of the time required for the disappearance of the blue color of the dye (methylene blue) is quantitatively added to milk. Reduction, *i.e.*, loss of color, is brought about largely by bacterial enzymes known as

reductases. Several hours are required for complete reduction by clean, milk, whereas a few minutes is sufficient for milk of poor quality.

Milks are graded according to the numbers of bacteria present, but and total solids and the cleanliness of production methods. Usual sanitary s ards require that **Grade A** milk be produced from tuberculin-negative an many instances, abortion or *Brucella*-free cattle, on farms that comply standards of cleanliness for dairies and by handlers free from infection. A terial plate count of not more than 30,000 organisms per ml. of milk at the of delivery is permitted. If pasteurized, a plate count of 200,000 bacteria ml. is permissible before pasteurization, although after pasteurization not than 30,000 viable organisms may be present.

**Certified milk**, introduced in 1892, represents the first attempt to pro safe milk. It is produced under the supervision of the American Associatio Medical Milk Commissioners, Inc., whose local representatives certify that milk is produced according to the rules. The cattle and handlers must be dise free, and the milk may not be over 36 hours old at time of delivery or con over 10,000 viable organisms per ml. Certified milk is now frequently pasteur a procedure which ensures its safety. The bacteria present in both Grad and certified milk are usually less than the permissible number, and a pasteurization very low counts may be obtained. **Grade B** milk is a relativ poor product which is rapidly being discontinued. It should always be p teurized.

**Food Poisoning.** The different kinds of food poisoning have little in co mon except that they result from ingestion of food containing the causat agent. They differ widely in etiology, clinical symptoms and treatment. Th are conveniently divided into the following three groups on the basis of etiology (1) chemical food poisoning; (2) poisoning due to toxic plants and anima and (3) bacterial food poisoning and food infection.

TABLE 21. CHEMICAL FOOD POISONING \*

CHEMICAL AGENT	SOURCE
Arsenic	Plants sprayed with lead arsenate
Antimony	Food prepared in poor quality gray enamel pans
Cadmium	Acid foods cooked in cadmium-plated utensils
Zinc	Acid foods cooked in galvanized utensils
Lead	Same as arsenic
Sodium fluoride	Cockroach powder, spilled into foods or mistaken baking powder

\* Adapted from Dack, G. M.: *Food Poisoning*, University of Chicago Press, 1943.

**Food Poisoning Due to Chemical Substances.** Food poisoning w chemical substances is uncommon and rarely is the cause of widespread o

<sup>1</sup> After Dack, G. M.: *Food Poisoning*, University of Chicago Press, 1943.



ks. Arsenic, antimony, cadmium, lead, mercury, zinc and certain other metals when eaten may give rise to food poisoning symptoms and epemics. Sodium fluoride, *i.e.*, cockroach powder, poisoning occurs occasionally. The metallic poisonings more often result from other causes than contamination of food. The major sources of chemical food poisoning are indicated in the preceding table.

Prevention of chemical food poisoning is largely individual and depends on avoidance of dangerous practices in preparation and storage of the food and chemicals in the home and restaurant. Education concerning proper methods of food handling is, therefore, a most important preventive measure. Supervision by the Pure Food and Drug Administration safeguards the purity of commercial products.

**Food Poisoning Due to Toxic Plants and Animals.** Some extremely poisonous substances are formed by higher plants. Among these are muscarine, "phallin," formed by inedible varieties of mushrooms, and ergot, produced by the fungus, *Claviceps purpurea*. Mushroom poisoning can best be prevented by eating only cultivated varieties, since edible wild mushrooms are identified with difficulty and should not be selected by an amateur. Symptoms of the disease include nausea, vomiting, thirst and later convulsions. Ergotism results from eating bread made of rye on which the fungus has grown. Ergot, a valuable medicine if properly used, when ingested in too large quantities causes itching, muscle cramps, gangrene and convulsions. The fungus and hence the disease have been prevalent only in Europe.

Other poisonings due to higher plants are sometimes encountered. Rhubarb (oxalic acid) poisoning results from using the leaves as cooked greens. Scurvy may result from eating fava beans or from inhaling pollen of the growing plants. The disease is an acute hemolytic anemia and is probably due to hypersensitivity to the plant. Milk sickness, or trematol poisoning, follows consumption of the milk of cows that have grazed on white snakeroot, a plant found in large numbers in the Southwestern United States. The symptoms include vomiting, constipation, profound weakness and later alkalosis.

Mussel (shellfish) poisoning has been reported from California and other coastal regions. This poison is probably elaborated by a certain protozoan (flagellate) on which the mussels feed. Ingestion of the mussels results in profound muscular weakness and paralysis, including respiratory paralysis. Outbreaks are seasonal, corresponding to the prevalence of the flagellate in coastal waters. Although epidemics sometimes occur, these diseases are relatively rare and often result from individual indiscretions. Therefore, education concerning dangerous higher plants and animals is an important means of control.

**Bacterial Food Poisoning.** Bacteria or their toxic products are the most frequent cause of food poisoning. Botulism, staphylococcal food poisoning and *Salmonella* food infection are the major diseases of this group, although a large number of pathogenic microorganisms may be conveyed by food. Botulism and

staphylococcal food poisoning are true intoxications due to ingestion of containing preformed bacterial toxins. The other diseases are generally considered to result from infection with living organisms. Because of the ever-present danger of contamination, food must be protected by cleanliness, proper processing and refrigeration.

**BOTULISM.** Botulism follows the ingestion of food containing the toxin of *Clostridium botulinum*. This organism is widely distributed in the soil throughout the world, and hence gains easy access to growing plants or pastured animals that are later used for food. Of the five known types of *Cl. botulinum*, Type A and B are chiefly responsible for human disease and Type E is occasionally involved.

Many types of food, including prepared sausages and canned meats, vegetables, fruits, seafood and milk, have been responsible for outbreaks of botulism. In Europe many outbreaks have been due to eating spoiled sausages—in the name, botulism, means sausage poisoning. In the United States canned foods, particularly home-canned products processed by the cold-pack method, have been most frequently involved. All of these foods provide the anaerobic conditions necessary for growth of the organism, having been insufficiently heated to destroy the heat-resistant bacterial spores. *Clostridium botulinum* is unable to grow in well aerated fresh foods and is destroyed by adequate processing methods.

When it grows in foods *Cl. botulinum* usually, but not invariably, produces a foul-smelling gas so that the food appears spoiled. Food suspected of being spoiled should **never** be tasted and should be destroyed, preferably by adding lye and burying the container and food. If salvaged, cans and jars should be thoroughly sterilized prior to reuse.

Botulism may be prevented by adequate canning methods. In food containing *botulinum* spores sometimes survive boiling for five and one-half hours, but they may be destroyed more rapidly at higher temperatures such as are obtained with steam under pressure. The survival time in canned foods is in part dependent on the penetration of heat through the cans and this, in turn, is affected by the consistency of the food and the size of the container. These factors are the basis for the processing times and temperatures used by commercial canners, the adequacy of which is attested by the rarity of botulism due to commercially canned foods.

These same principles of heating under pressure for sufficient time and at a high enough temperature to kill botulinum spores may be applied to home canning. The cold-pack method is inadequate because it does not kill the spores. On the other hand, the recommended pressure cooker method allows prolonged heating by steam under pressure. Details of this method can be obtained from cooking schools and a recent bulletin on safe canning practices issued by the United States Department of Agriculture. Despite wide publicity, this method has not yet been universally adopted. It is hoped, however, that further education will speed conversion to safe canning methods. As



ed safeguard, canned food may be boiled for fifteen minutes before tasting.

In the event that incriminated food is ingested, antitoxin should be administered as soon as possible.

**STAPHYLOCOCCAL FOOD POISONING.** Staphylococcal food poisoning is also caused by the ingestion of preformed bacterial toxin. Although staphylococci are almost universally distributed, only some of these organisms produce enterotoxin. Such strains are usually unidentified unless responsible for illness.

Many foods have been responsible for outbreaks of staphylococcal food poisoning and more are constantly being incriminated. They include meat products (such as cured meats), bakery goods (especially cream-filled pastries), ice cream, cheese, sandwiches and gravies. When contaminated, there is usually no change in appearance, odor or taste of the food that would indicate presence of the bacteria. At least several hours are required for the production of enterotoxin in food, although the time is variable and dependent upon the size of the inoculum, the temperature and the type of food. Involved foods are commonly found to have been prepared many hours, even days, prior to eating. Freshly prepared foods are usually safe; foods involved in outbreaks are often eaten soon after preparation without causing ill effects.

Although it is undoubtedly the most frequent type, staphylococcal food poisoning is usually identified only when many persons are affected in a single outbreak. The disease is most often mild, and after an acute gastro-intestinal upset, characterized by nausea, vomiting and diarrhea, recovery is complete. In severe cases, however, symptoms are severe, and the patient is incapacitated for several days. The illness rarely terminates fatally. Because the disease is usually mild, many individual cases are unidentified.

Staphylococcal food poisoning may be prevented by prompt and continuous refrigeration of food. Prepared foods should not be stored at room temperature. Cans, ice cream cans, pastry bags and the like should be carefully cleaned and well as sterilized, in order to remove any small bits of food on which a "starter" culture might develop. An effective reheating process has been devised to destroy staphylococci in custard-filled pastries, before production of toxin has occurred. Persons having *Staphylococcus* infections, such as furuncles, should not work as food handlers, and general cleanliness should be observed. Since it is remarkably heat resistant, staphylococcal enterotoxin, once formed, is not effectively destroyed by the usual methods of reheating food.

**SALMONELLA FOOD INFECTION.** *Salmonella* food poisoning, unlike botulism and staphylococcal food poisoning, is due to infection with living organisms. The species most frequently incriminated are *Salmonella typhimurium* and *Salmonella enteritidis*. These and related members of the group encountered in food poisoning outbreaks are pathogenic for a wide variety of animals, including cattle, sheep, swine, rats and mice, all of which may be sources of infection for man. Recently human carriers of these organisms have been identified. Consequently meat and meat products, and milk and milk products from infected

animals or food contaminated by rodents and human carriers may give rise to this disease. Pasteurized milk and thoroughly cooked or canned foods are usually safe. Contaminated food appears normal.

Symptoms of illness most often begin 12 to 24 hours after ingestion of contaminated food, and consist of fever, prostration, malaise, nausea, vomiting, abdominal pain and diarrhea. Death sometimes occurs.

Control of *Salmonella* food infection includes exclusion of diseased animals from food, pasteurization of milk and milk products, thorough cooking of food, examination of food handlers and protection of food from rodents and flies. Federal inspection of meat and other products, rat-proof construction of food storage places and supervision of carriers are important parts of the control program. Finally, education regarding the danger of sick animals as a source of food is essential. Patients in the hospital should be isolated.

**OTHER FOOD-BORNE INFECTIONS.** A number of additional infections may be transmitted by food. Typhoid and dysentery carriers or patients may contaminate food, and respiratory pathogens, such as streptococci, are at times transferred in food. In these instances the cook or other food handler often is the source of infection. Certain parasitic infections, such as trichinosis and intestinal fluke and worm infections, result from eating improperly cooked food containing infectious parasites.

Sanitary preparation of food, refrigeration, supervision of food handlers, exclusion of flies and thorough cooking afford protection against these diseases as well as against food poisoning.

**Sanitation of Eating and Drinking Utensils.** Pathogenic organisms present in saliva and sputum may be transferred on improperly washed dishes and utensils. Several thousands to millions of bacteria are frequently cultured from plates, glasses and silverware after improper cleansing, so that pathogenic bacteria may well be present. On the other hand, counts of less than 100 bacteria are commonly obtained from properly washed dishes. Observation of dishwashing methods in many restaurants indicates the need for improvement of existing practices.

Adequate methods of sanitation are described in the Ordinance and Code Regulating Eating and Drinking Establishments recommended by the U. S. Public Health Service. Dishes should be carefully scraped, preferably pre-rinsed, washed in clean, hot, soapy water and finally rinsed in a bactericidal solution such as well-chlorinated or scalding water, for 2 minutes. Dishes that are washed in this way and are not handled excessively after washing yield counts well below the standard of 100 bacteria. Provided the dishes are conscientiously washed, hand methods of dishwashing are satisfactory. Machine methods are, however, usually more satisfactory for institutions. Dishes and utensils used by patients with communicable diseases should be boiled or steam-sterilized. In addition to sterilization, careful cleansing and the removal of food particles are important.

Dishwashers too often are not aware of the importance of their job, so that education concerning disease transmission by improperly washed dishes and



ation of proper methods of sanitation are necessary. Schools for restaurant personnel are being conducted in many cities as a part of the public health program.

## VENEREAL DISEASES

The venereal diseases form a group of infections of bacterial, spirochetal and etiologic that are commonly transmitted through sexual intercourse. Nevertheless, they may also be transmitted congenitally, by direct contact with infectious skin lesion and by indirect contact with objects, such as dressings and linens, recently used by infected persons. Congenital transmission of syphilis from the untreated mother to the child *in utero* is a major problem. Postnatal infection by contact other than venereal, although well established, is relatively infrequent in all these diseases.

The diseases included in this group are syphilis, gonorrhea, chancroid, lymphogranuloma (lymphogranuloma) venereum and, probably, granuloma inguinale. The most widespread of these are syphilis and gonorrhea, and the following discussion will be limited to these two diseases. Nonetheless, the protective methods described apply in general to the other members of the group as well.

**Incidence.** The incidence of syphilis and gonorrhea is not accurately known despite the fact that they, like the other communicable diseases, are reportable. Reporting is, however, very incomplete, and many patients do not come under medical supervision, owing in part to the superficial mildness and insidious nature of their infections. Data obtained from serological tests on selective service registrants and other surveys indicate that the incidence of syphilis varies from less than 1 to as much as 43 per cent in different groups of the population. The usual estimate is about 10 per cent. The incidence of gonorrhea cannot be estimated with reasonable accuracy. Familial transmission of gonorrhea and syphilis is, of course, frequent. Syphilis is relatively infrequent among professional and similar groups. It is relatively frequent among Negroes.

**Detection of Cases.** Detection of cases of venereal disease is of primary importance to the control of these infections, and it is at the same time a major problem. The investigation of contacts, family and otherwise, and treatment of infections thus discovered is best carried out by trained personnel. The investigation must be tactfully and intelligently conducted. Serological tests for syphilis are now a part of prenatal and general clinic and hospital care and provide one excellent means of case finding. The detection of syphilis in expectant mothers and the initiation of treatment during pregnancy is of particular importance, since it not only benefits the mother but reduces the number of stillbirths due to syphilis and disease in the child. The earlier treatment is begun, the more satisfactory are the results in both mother and child. Children of syphilitic mothers, however, should be medically supervised and tested serologically for some years after birth. Premarital examinations for both syphilis and gonorrhea are required by an increasing number of states and contribute greatly to the reduction of these diseases.

**Control.** Control of the venereal diseases is an urgent public health problem that compares in magnitude with those of the acute respiratory diseases and tuberculosis. It may be divided into educational, social, medical and legal.

Education of the public regarding the nature and importance of these diseases and their treatment is carried on by means of posters, literature, films and motion pictures. Understanding of the problem by the patient leads to his cooperation in case detection, treatment and follow-up examinations. Emphasis should be placed on the infectious nature of these diseases, their danger of transmission and the availability and benefits of treatment. The prevalence of infection and, therefore, the danger of acquiring disease through contact, particularly sexual, with fresh infectious material should be pointed out, as should the particularly high incidence associated with prostitution and delinquency. Educational material must be carefully chosen for presentation and suited to the persons receiving information. An educational program may be carried out in schools or through other organizations and should begin during adolescence.

A number of legal control measures are employed against the venereal diseases, including the laws requiring registration of cases, Credé or mercuric nitrate treatment of the eyes of newborn babies, the premarital examination and the anti-vice laws. Among the latter, the attempts to eliminate prostitution and to care for cases of sex delinquency are of particular importance. The development of supervised group recreational activities is an important part of venereal disease control.

Medical measures include detection and treatment of cases, provision of clinic and hospital facilities and personnel, the isolation of infectious individuals and follow-up activities. Prompt and adequate treatment rapidly sterilizes lesions of syphilis and gonorrhea, so that the patient can after a short time resume his usual activities without constituting a source of infection for others. Further treatment can then be carried out in the clinic. The patient must refrain from personal contact with others and must use individual bed linen, towels and utensils during infectious stages. Utensils, instruments and linens must be sterilized as in the other infectious diseases. The gonorrhea patient should be warned that uncleanly personal hygiene may result in the infection of his eyes. Personnel caring for infectious patients should use glove and gown technique.

Federal and local campaigns against the venereal diseases have been greatly expanded during recent years through cooperative effort in diagnosis, treatment, education and research.



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## INSECT-BORNE DISEASE

Diseases spread by insects, such as mosquitoes and other arthropod vectors, ticks, mites, etc.—are among the most important and widespread infectious diseases. In addition to causing large numbers of deaths, they are responsible for long-term disability, illness and economic loss. The organisms that may be transmitted by insects include bacteria, viruses, rickettsiae, protozoa and certain fungi.

In order for living vectors to transmit infection a chain of events must be successfully completed. First, the parasite must be present and the arthropod must be susceptible to infection with it. Second, the vector must habitually feed on the particular hosts of the microorganism. To illustrate: malaria is transmitted effectively only when anopheline mosquitoes which feed largely or exclusively on man become infected. In a malarious area one species of anopheline mosquito is often found to be chiefly responsible for the maintenance of the disease, although several species of mosquitoes may abound in the region. In the absence of malaria this same mosquito, because uninfected, will be unable to transmit parasites. Similarly, yellow fever no longer occurs in many parts of North America because cases do not exist from whom the mosquito, *Aedes triseriatus*, can become infected. On the other hand, pathogenic organisms are often transmitted hereditarily in ticks from one generation to the next, so that, once they enter a region, the organisms causing spotted fever and tularemia, for example, can be continuously harbored by the vector.

Aside from mechanical transmission, e.g., the dissemination of typhoid fever by flies, a parasite may undergo one of several types of development in the vector. It may multiply in the vector, as does the plague bacillus (*Pasteurella pestis*) in the flea, or it may go through a phase of its life cycle as does the malarial parasite in the mosquito. The malaria parasite also increases in number in the insect. On the other hand, the filarial worms develop in the insect without increasing in number. Some of the more important human diseases transmitted by arthropods are listed in Table 22.

Control measures against insect-borne disease may be directed against the parasite, the vector or both. The methods used to accomplish these objectives are included in the following general categories: (1) treatment of cases and carriers; (2) control of the vector; (3) control of lower animal hosts; (4) per-

TABLE 22. IMPORTANT HUMAN DISEASES TRANSMITTED BY ARTHROPODS

VECTOR	DISEASE	CHIEF GEOGRAPHICAL DISTRIBUTION
MOSQUITOES: <i>Anopheles</i>  <i>Culex</i> <i>Aedes</i>	Malaria / <i>downia</i> Filariasis (elephantiasis) Yellow fever  Dengue fever Equine encephalomyelitis	Widespread in tropics and subtropics. ( <i>Schools</i> ) Tropics and subtropics. Central and South America West Africa, West India Tropics and subtropics. Widespread.
FLIES: Houseflies ( <i>Musca domestica</i> )  Deer flies ( <i>Chrysops discalis</i> ) Tsetse flies ( <i>Glossina sp.</i> )  Sand flies ( <i>Phlebotomus sp.</i> )	Typhoid fever, paratyphoid fever, dysentery Asiatic cholera Tularemia  African sleeping sickness (trypanosomiasis)  Pappataci fever Verruga peruviana  Leishmaniasis (Oriental sore, kala-azar)	World-wide.  India and Asia. United States (western).  Africa (West Africa, the Congo, Rhodesia, the Sudan). Mediterranean region, India, Ceylon, South China. South America (Chile, Peru, Ecuador, Bolivia). Mediterranean region, China, India, parts of the Congo
BUGS: Bedbugs ( <i>Cimex lectularis</i> ) Kissing bugs or reduviids ( <i>Triatoma megista</i> )	Not an important vector of disease Chagas' disease (South American trypanosomiasis)	South and Central America
LICE: <i>Pediculus humanus</i>	Epidemic typhus fever Relapsing fever	Widespread, chiefly Europe Widespread.
FLEAS: Rat flea ( <i>Xenopsylla cheopis</i> ) <i>Ceratophyllus sp.</i> and other fleas of wild rodents	Bubonic plague Sylvatic plague, endemic (murine) typhus fever	Orient, India, South America, western United States Widespread.
TICKS: Wood ticks, dog ticks, and rabbit tick ( <i>Dermacentor</i> and <i>Haemaphysalis sp.</i> )  <i>Ornithodoros sp.</i>	Spotted fever Tularemia  Equine encephalomyelitis Colorado tick fever Relapsing fever	United States. United States.  United States. United States. East Africa, upper Congo, western United States.
MITES: <i>Trombicula</i>	Tsutsugamushi disease  Rickettsialpox	Japan, Malaya, Formosa, Sumatra. United States.



al or individual protection. A combined attack is frequently made against any disease.

**Treatment of Cases and Carriers.** Specific therapeutic agents effective in these diseases have been discussed previously. The community benefits from the treatment of patients by the elimination of sources of infection, although better protection of a large population is generally obtained at lower cost by measures directed against the vector. In malaria, drug prophylaxis with quinine or pamaquine, pentaquine, chloroquine, etc., is widely used for the prevention of symptoms. It is of inestimable value to troops and others in malarious regions. However, it should be realized that infection is not prevented by drug prophylaxis if it is suppressed, so that symptoms may appear when the drug is discontinued. Extensive treatment is then necessary to eliminate the infection.

**Control of the Vector.** Arthropod vectors are seldom completely eliminated by campaigns against them, but they may be so reduced in number that they no longer effectively transmit disease. The attack may be made against (1) the adult arthropod; (2) vulnerable larval stages; and (3) breeding places. The methods depend upon knowledge of both the principles of control and the local situation and are different for different vectors.

**Mosquito Control.** The control of adult mosquitoes is at best only partially successful. Screening of houses, use of bed nets and insecticides, such as pyrethrum, are of some value and should be employed in a region infested with mosquitoes. Recently the insecticide DDT has come into use and promises to be successful against mosquitoes.

Mosquito larvae, all of which live in water, are vulnerable to a variety of chemical poisons and certain larva-eating fish (*Gambusia*). In addition, it is often possible to eradicate the breeding places. Chemical poisons, such as oil emulsions, Paris green (an arsenical), DDT and phenol mixtures effectively destroy mosquito larvae, but must be reapplied to the water at frequent intervals to ensure protection. They are inexpensive. Devices including individual sprays and dusting apparatus, mechanical bubblers and specially equipped airplanes are used to apply larvicides. Destruction of mosquito breeding places by swamp drainage and other drainage projects often is an expensive and difficult engineering program. Large projects have, however, been successfully carried out in many areas, and small clean-up campaigns are an essential part of mosquito control.

Initiation of control measures should be preceded by a survey of the area to determine the responsible mosquitoes and their breeding places. The survey is particularly important in malaria control, since anopheline mosquitoes breed in every conceivable water habitat. Some species lay eggs in fast-flowing freshets, whereas others breed in sluggish streams, swamps or brackish sea estuaries. The breeding places of *Aedes aegypti*, the yellow fever mosquito, are relatively easily identified, because this mosquito breeds in tin cans, vases and similar house-water containers. It is often called the domesticated mosquito.

***Fly Control.*** The domestic house fly breeds in manure, garbage and the like. Sanitary disposal of human and animal waste products is, therefore, an extremely important means of fly control. Manure should be promptly spread upon fields or protected from flies; and privies, septic tanks and garbage cans should be fly-proof. Buildings should be adequately screened and fly paper and the swatter should be used unsparingly.

Deer flies and related species are controlled largely by individual measures. Tsetse flies present particularly difficult and important control problems. Because these insects give birth to living larvae which are deposited individually in shaded, moist and sandy regions, control measures such as those effective against mosquitoes are unsuccessful. Destruction of favorite shaded areas that shelter the flies and trapping are the measures generally applied, but these are only partially successful. On the other hand, sand flies are best controlled by eliminating the damp shady crevices in rocky deposits and the cracks in walls that serve as breeding places.

***Control of Bed Bugs.*** Extensive infestation with these parasites requires fumigation of the building with hydrogen cyanide or sulfur. The quarters must be vacated during fumigation, which is best conducted by professional fumigators. Less extensive infestation may be eradicated by applying corrosive sublimate (mercuric chloride), steam or insecticidal sprays. A blow torch is an effective means of destroying adults and eggs on metal springs and bedsteads. If chemicals are used suitable methods of application and protection should be observed.

***Control of Fleas, Ticks and Mites.*** Fleas, ticks and mites are best controlled indirectly by control of the lower animal hosts or by personal protective measures. Fleas, however, sometimes infest houses from which they may be eradicated by fumigation and application of insecticide.

***Control of Lower Animal Hosts.*** Rodents, particularly rats, are the animal reservoirs of bubonic plague and endemic typhus fever, both of which are transmitted by fleas. Control measures against these animals are centered in disease-infested areas, and have as objectives (1) eradication of the rodents, (2) exclusion of the rodents from buildings; and (3) prevention of spread to previously uninfested areas. Rats may be destroyed by trapping, fumigation and poisoning. The last method should be used with caution, however, since human illness may result from improper agents or methods of application. Ships from plague-infected ports are subject to quarantine, inspection, fumigation, and the use of rat-proof barriers on hawsers to the dock.

Buildings made of concrete, stone, metal and fine wire mesh are resistant to gnawing, provide protection against burrowing and provide the best protection against rats. Such construction not only reduces rat harborage, but also curtails the rodent's food supply when applied to barns, grain cribs and elevators.

Control of wild rodents, such as ground squirrels, presents even greater difficulties but, nonetheless, must be undertaken when these animals constitute



ance to the human population. The methods include shooting, trapping, poisoning and the fumigation of burrows.

Tick-borne disease is better controlled by methods other than those directed against the lower animal hosts. Cattle are, however, dipped in a solution of kerosene to eradicate the tick vectors of Texas cattle fever. The numbers of ticks can also be reduced by grazing sheep on heavily infested areas or by burning the area.

**Personal Protective Measures.** Personal protective measures are of primary importance against lice, ticks and mites and are of value against other arthropods.

The control of epidemic (louse-borne) typhus fever is largely dependent upon widespread use of personal delousing methods. The body should be thoroughly completely bathed, preferably with a cresol soap. Head lice and their eggs should be destroyed by treating the hair and scalp with a kerosene-vinegar solution, 1 per cent solution of tincture of larkspur or other suitable insecticide followed by thorough washing. Lice and eggs on clothing are killed by civilian laundry or dry cleaning methods, and personal cleanliness is usually sufficient protection against infestation. In the presence of disease or in the field additional methods should be used. Armies maintain delousing stations at which baths with insecticidal solutions and fumigation or heat sterilization of clothing and equipment are provided. Hair clipping is sometimes necessary. Personnel engaged in typhus control should wear protective clothing and should employ rigid personal hygiene. Camp or hospital bed rolls are to be preferred to hotel equipment in grossly infested areas. Dusting powder containing DDT is effective against fleas and lice. Endemic areas of tick-borne disease should be avoided insofar as possible, and individuals who enter them should wear protective clothing. Woodsmen, tourists and hikers should remove any attached ticks as soon as possible.

Vaccination, when available, and drug prophylaxis should be utilized by persons exposed to unusual risks. Vaccination is effective against plague, spotted fever, typhus fever and typhoid fever, and drugs are used chiefly to control malaria.

Insecticides and insect repellents are of some value as a means of personal protection.





# Appendix

## EARLY MILESTONES IN MICROBIOLOGY

- BACON, ROGER (1214-1294), clarifies principles of optics; lays foundation for simple microscope and convex spectacles.
- FRACASTORO, GIROLAMO, publishes books on contagious diseases in which he speaks of *seminaria* (seeds or germs) of disease.
- ?) Compound microscope invented. HANS and ZACHARIAS JANSSEN generally credited with its invention.
- GALILEO devises a compound microscope.
- HOOKE, ROBERT, publishes *Micrographia* in which he records observations on microscopic structure of plants and describes cells for the first time.
- REDI, FRANCESCO, demonstrates generation of maggots from flies' eggs; disproves spontaneous generation of insects.
- LEEUEWENHOEK, ANTONY VAN (1632-1723), writes first letter to Royal Society describing microscopic appearance of mold and structures of the bee and louse as seen through his simple microscope.
- LEEUEWENHOEK discovers "animalcules" (protozoa and possibly bacteria).
- LEEUEWENHOEK observes yeast "globules" in beer.
- LEEUEWENHOEK describes and draws figures of bacteria.
- Variolation against smallpox (human inoculation of material from smallpox lesions) practiced in Constantinople.
- MONTAGU, LADY MARY, introduces variolation in England; has son inoculated against smallpox.
- JOBLLOT, LOUIS, confirms existence of "animalcules"; his experiments with heated infusions refute theory of spontaneous generation.
- LINNAEUS publishes *Systema naturae* (classification of animals) in which "animalcules" are classified under "Vermes" (worms) in class "Chaos."
- 76 SPALLANZANI, LAZZARO, shows that microorganisms will not appear in hermetically sealed, heated infusions; concludes theory of spontaneous generation is untenable.
- MÜLLER, OTTO FRIEDRICH, attempts classification of protozoa and bacteria.
- JENNER, EDWARD, inoculates William Phipps with material from cowpox lesions on arm of milkmaid Sarah Nelmes (May 14). Proves **vaccination** protects against smallpox.
- APPERT, NICHOLAS, introduces process for canning foods.
- AMICI of Modena first corrects false colors (chromatic aberration) and defective definition (spherical aberration) in the compound microscope following principles laid down by Hall (1733) and Dolland (1757) for the telescope. Works with objective immersed in water and other liquids.
- BROWN, ROBERT, describes brownian movement of microscopic bodies.
- LISTER, J. J., father of Baron Joseph Lister, perfects an achromatic microscope; begins darkfield microscopy.

- 1835-36 BASSI, AGOSTINO, recognizes a mold as the cause of a disease of silkworms (*muscardine* or *mal del segno*). Microbic etiology of a disease determined the first time.
- 1836-37 SCHWANN, THEODOR, devises ingenious experiments to disprove spontaneous generation; proves it is not the air, but something in the air, that causes putrefaction.
- 1836 CAGNIARD-LATOUR, CHARLES, shows yeast to be a microorganism; states that yeast causes fermentation.
- 1837 SCHWANN, THEODOR, independently discovers yeast; names it "*Zuckerpilz*" (sugar fungus) and relates it to fermentation.
- 1837 KÜTZING, FRIEDRICH T., independently discovers and describes yeast cell; states that fermentation is a vital process.
- 1838 SCHLEIDEN, MATTHIAS J., describes plant cells.
- 1838 EHRENBERG, CHRISTIAN G., classifies "Infusoria," including protozoa and bacteria, with other microscopic animals.
- 1839 SCHWANN, THEODOR, discovers cells of animal tissues; publishes treatise on cell theory.
- 1839 SCHÖNLEIN, JOHANN L., discovers fungus of favus (*Achorion schönleini*).
- 1840 HENLE, FRIEDRICH G., outlines the facts which must be determined in order to prove the germ theory of communicable disease.
- 1841-42 BERG, F. T., and GRUBY, DAVID, independently discover *Oidium albicans* (*Candida albicans*).
- 1843 HOLMES, OLIVER WENDELL, points out contagious nature of puerperal fever.
- 1843-44 GRUBY, DAVID, discovers *Microsporum audouini* and *Trichophyton tonsurans*, causes of ringworm.
- 1847 SEMMELWEIS, IGNAZ P., discovers pathogenesis of puerperal fever; advocates that those in obstetrical practice wash hands in chloride of lime solution to prevent this disease.
- 1848 PASTEUR, LOUIS (1822-1895), studies nature of tartaric acid. Discovers molecular asymmetry; dextro- and levo-rotary forms of tartrate crystals.
- 1849 SNOW, JOHN, publishes data proving cholera is water-borne. Later traces great London epidemic of 1854 to the Broad Street pump.
- 1849 POLLENDER, FRANZ A. A., discovers microscopic, rod-shaped bodies in blood and spleen of cows dead from anthrax.
- 1850 RAYER, PIERRE F. O., and DAVAIN, CASIMIR J., observe rod-shaped bodies in blood of sheep dead from anthrax; transmit disease by inoculating anthrax blood into healthy sheep.
- 1850-56 WENHAM, FRANCIS H., first employs oil immersion lens.
- 1854 COHN, FERDINAND, recognizes plant nature of bacteria.
- 1854 SCHRÖDER, HEINRICH G. H., and DUSCH, THEODOR VON, filter air through cotton wool to prevent putrefaction of heated infusions.
- 1856 BUDD, WILLIAM, suggests typhoid fever is transmitted by sewage-contaminated water.
- 1856 PERKIN, WILLIAM H., prepares the first aniline dye (aniline purple) from coal tar.
- 1857 Typhoid fever traced to milk in Penryth, England.
- 1857 NÄGELI, CARL W. VON, suggests the name *Schizomycetes* (fission fungi) for bacteria.
- 1857 PASTEUR discovers lactic acid fermentation is a microbic process.
- 1860 PASTEUR completes studies proving that yeast is an organism which ferments sugar to form alcohol and  $\text{CO}_2$ .
- 1860 LEMAIRE, JULES, points out the antiseptic properties of carbolic acid.



- 51 PASTEUR demonstrates bacteria in air; disproves spontaneous generation.  
 PASTEUR studies butyric acid fermentation; discovers that fermentation is an anaerobic process; recognizes anaerobic microorganisms.  
 PASTEUR shows that acetic acid fermentation is due to microorganisms.
- SCHULTZE, MAX, defines protoplasm and the cell.
- 58 DAVAINÉ's extensive research confirms his belief that anthrax is caused by the "bacteridia" observed in cases of this disease.
- MENDEL, GREGOR, publishes studies on plant hybrids; establishes principles of heredity and science of genetics.
- VILLEMIN, JEAN A., demonstrates transmissibility of tuberculosis by inoculation.
- 70 PASTEUR investigates silkworm disease (pebrine); demonstrates its microbic origin.
- PASTEUR publishes *Études sur le vin* dealing with "diseases" of wines due to microorganisms and the prevention of spoilage by heating wine.
- LISTER, JOSEPH, introduces antiseptic surgery.
- PASTEUR publishes studies on microbiology of vinegar (*Études sur le vinaigre*).
- HOFFMANN, HERMANN, uses aqueous solutions of carmine and fuchsin to stain bacteria.
- ABBÉ, ERNST, manufactures substage condenser of the compound microscope.
- WENHAM, FRANCIS H., demonstrates use of cedar oil with immersion lens before Royal Microscopical Society.
- WEIGERT, CARL, uses methyl violet stain to reveal bacteria in tissues.
- COHN, FERDINAND, classifies bacteria, recognizing different species, constancy of form in bacterial species and physiologic variation in morphologically similar bacteria.
- BREFELD, OSCAR, uses gelatin medium to obtain pure cultures of higher fungi.
- HANSEN, G. H. ARMAUER, discovers the leprosy bacillus.
- OBERMEIER, OTTO H. F., discovers the spirochete of relapsing fever.
- EHRlich, PAUL, introduces dried blood smears and improves staining methods.
- LÖSCH, F., describes *Endamoeba histolytica* and demonstrates its pathogenicity.
- PASTEUR publishes fermentation studies on beer (*Études sur la bière*).
- KOCH, ROBERT (1843-1910), proves cause of anthrax; obtains pure cultures of anthrax bacillus; describes its life cycle and resistance of its spores.
- 77 TYNDALL, JOHN, observes that heated infusions remain sterile in "optically empty" air. Final blow to theory of spontaneous generation.
- BURRILL, T. J., discovers bacteria of pear blight.
- TYNDALL concludes that bacteria may exist in two forms, one thermolabile and one thermostable; introduces fractional sterilization (Tyndallization).
- COHN independently observes the thermolabile, vegetative stage and the thermostable spore stage of the hay bacillus (*B subtilis*).
- KOCH fixes bacteria in dried films for microscopic examination; improves staining methods.
- PASTEUR discovers anaerobic, spore-bearing pathogen *Vibrio septique* (*Clostridium septicum*).
- 81 EHRlich perfects method of staining blood films. Introduces use of methylene blue.
- WEIGERT recommends aniline dyes (methyl violet, fuchsin, aniline brown) for staining bacteria.
- ABBÉ manufactures effective oil immersion lens.
- KOCH proves bacterial etiology of wound infections.
- LISTER, JOSEPH, uses dilution method to obtain pure cultures of bacteria.
- NEISSER, ALBERT, discovers the gonococcus.

- 1879 MANSON, PATRICK, discovers mosquito transmission of filariasis, a disease caused by a microscopic worm (*Filaria bancrofti*). First recognition of insect-borne disease.
- 1880 PASTEUR immunizes against chicken cholera using attenuated (aged) cultures.
- 1880 EBERTH, CARL J., discovers the typhoid bacillus.
- 1880 EVANS, G., discovers the first pathogenic trypanosome, *T. evansi*, in the blood of horses affected with surra.
- 1880 LAVERAN, ALPHONSE, discovers protozoan parasite of malaria.
- 1881 PASTEUR produces successful vaccine for anthrax using organisms attenuated by cultivation at 42° C.
- 1881 KOCH introduces simple method for obtaining pure cultures of bacteria by streaking solid, nutrient gelatin medium.
- 1881 OGSTON, ALEXANDER, discovers micrococci (staphylococci and streptococci) in pus from abscesses.
- 1881 PASTEUR discovers the pneumococcus.
- 1881 STERNBERG, GEORGE M., independently discovers the pneumococcus.
- 1882 KOCH discovers the tubercle bacillus. Defines "Koch's postulates."
- 1882 HESSE, FRAU WALTHER, substitutes agar-agar for gelatin in Koch's bacteriological culture media.
- 1883 KOCH discovers the vibrio of Asiatic cholera.
- 1883 KOCH introduces pour plate method for isolating bacteria.
- 1883 KLEBS, T. A. EDWIN, describes the diphtheria bacillus.
- 1884 CREDÉ, CARL S. F., introduces instillation of silver nitrate into eyes of newborn to prevent ophthalmia neonatorum.
- 1884 METCHNIKOFF, ÉLIE, states phagocytic theory of immunity.
- 1884 GAFFKY, GEORG, obtains pure culture of the typhoid bacillus.
- 1884 LÖFFLER, FRIEDRICH A. J., obtains pure culture of the diphtheria bacillus.
- 1884 GRAM, CHRISTIAN, introduces his method of differential staining of bacteria.
- 1885 BERTHELOT, T., discovers microorganisms in soil cause nitrogen fixation.
- 1885 PASTEUR develops vaccination against rabies.
- 1886 BERGMANN, ERNST VON, introduces steam sterilization in surgery.
- 1886 ESCHERICH, THEODOR, discovers the colon bacillus; names it *Bacillus coli commune*.
- 1886 SALMON, DANIEL E., and SMITH, THEOBALD, immunize animals against hog cholera by inoculating heat-killed hog cholera bacilli.
- 1886 HELLRIEGEL, H., and WILFARTH, H., discover nitrogen-fixing bacteria living symbiotically with leguminous plants.
- 1887 BRUCE, DAVID, discovers cause of Malta fever (*Brucella melitensis*).
- 1887 WEICHELBAUM, ANTON, discovers and isolates the meningococcus.
- 1887 PETRI, RICHARD J., introduces the petri dish.
- 1888 GÄRTNER, A., isolates *Bacillus enteritidis* (*Salmonella enteritidis*) from meat responsible for a food poisoning outbreak.
- 1888 ROUX, P. P. ÉMILE, and YERSIN, ALEXANDRE E. J., discover diphtheria toxin.
- 1888 NUTTALL, GEORGE H. F., notes bactericidal power of the blood.
- 1889 DUCREY, AUGUSTO, discovers the bacillus of chancroid (*Hemophilus ducreyi*).
- 1889 KITASATO, S., obtains pure cultures of the tetanus bacillus; proves it is the cause of lockjaw.
- 1890 BEHRING, EMIL VON, and KITASATO discover diphtheria and tetanus exotoxins.
- 1890 KOCH introduces tuberculin; notes tuberculous animals resist reinoculation.
- 1890 WINOGRADSKY, S., isolates nitrifying bacteria from soil; demonstrates their relation to soil fertility.
- 1890 MILLER, W. D., publishes *The Micro-organisms of the Human Mouth*; pioneer in dental bacteriology.



- WOLFF, F. M., and ISRAEL, J., isolate pathogenic *Actinomyces*.
- HAISTED, WILLIAM S., introduces use of rubber gloves in operative surgery.
- BERGMANN, ERNST VON, standardizes general aseptic ritual in surgery.
- NORDTMAYER, H., invents the Berkefeld filter made of diatomaceous earth (*Kieselguhr*) taken from the Berkefeld mine.
- SMITH, THEOBALD, and KILBORNE, F. L., demonstrate tick transmission of Texas cattle fever.
- WELCH, WILLIAM H., and NUTTALL, G. H. F., isolate the gas gangrene bacillus (*Clostridium perfringens*; *Clostridium welchii*).
- IWANOWSKI, D., discovers filter-passing agent of tobacco mosaic disease; demonstration of first filtrable virus.
- SMITH, ERWIN, investigates bacterial diseases of plants.
- BRUCE, DAVID, discovers trypanosome of nagana (*T. brucei*).
- ERMENGEN, ÉMILE P. M. VAN, discovers the anaerobic, sporulating bacillus of botulism.
- YERSIN and KITASATO independently discover the bacillus of plague.
- PFEIFFER, AUGUST, discovers bacteriolysis, lysis of the cholera vibrio in immune animals (Pfeiffer's phenomenon).
- ZIEHL-NEESEN method of staining tubercle bacillus introduced by FRANZ H. R. ZIEHL and FRIEDRICH C. A. NEESEN.
- GRUBER, MAX, and DURHAM, HERBERT, discover bacterial agglutination.
- WIDAL, G. FERNAND L., introduces agglutination test for typhoid fever.
- WRIGHT, ALMROTH E.; PFEIFFER, A., and KOLLE, W., vaccinate against typhoid fever.
- KRAUS, RUDOLF, discovers precipitating property (precipitins) in immune serum.
- FLÜGGE, CARL, states theory of droplet infection.
- EHRlich states side-chain theory of immunity. Establishes principles of standardization of toxins and antitoxins.
- BUCHNER, EDOUARD, separates fermentative enzyme "zymase" from yeast cells.
- BANG, B. L. F., discovers cause of contagious abortion in cattle (*Brucella abortus*).
- BEIJERINCK, MARTINUS, independently discovers the filtrable virus of tobacco mosaic disease.
- GILCHRIST, R. C., and STOKES, W. R., isolate fungus of North American blastomycosis (*Blastomyces dermatitidis*).
- ROSS, RONALD, demonstrates development of parasite of avian malaria in the mosquito.
- SHIGA, KIYOSHI, discovers bacillus of dysentery (*Shigella dysenteriae*).
- BORDET, JULES, discovers hemolysis, i.e., lysis of red blood cells by specific immune serum; finds immune serum contains a heat-labile factor (alexin; complement) and a heat-stable factor.
- LÖFFLER, FRIEDRICH, and FROSCHE, PAUL, discover filter-passing virus of foot and mouth disease; first demonstration of animal virus.
- GRASSI, B.; BIGNAMI, A., and BASTIANELLI, G., show that *Anopheles* mosquito transmits human malaria.
- OPHULS, W., and MOFFITT, H. C., show that the cause of coccidioidomycosis is a fungus (*Coccidioides immitis*).
- REED, WALTER; CARROLL, JAMES; LAZEAR, JESSE W., and AGRAMONTE, A., demonstrate mosquito transmission of yellow fever.
- LEISHMAN, WILLIAM B., and DONOVAN, CHARLES, discover the protozoan of kala-azar.
- DEVRIES, HUGO, states mutation theory.

- 1901-02 DUTTON, J. E., and FORDE, R. M., discover trypanosome of African sleeping sickness (*T. gambiense*).
- 1901 BORDET, JULES, and GENGOU, OCTAVE, demonstrate complement fixation.
- 1901 LANDSTEINER, KARL, discovers blood groups (iso-agglutination).
- 1902 PORTIER, P., and RICHET, C., describe and name anaphylaxis.
- 1903 BRUCE, D., demonstrates transmission of sleeping sickness by the tsetse fly.
- 1903 WRIGHT, A. E., and DOUGLASS, STEWART, show that immune serum contains specific substance (opsonin) which enhances phagocytosis.
- 1903 ARTHUS, M., produces local anaphylaxis (Arthus phenomenon).
- 1905 SCHAUDINN, FRITZ, and HOFFMANN, ERICH, discover the spirochete of syphilis.
- 1905 BORDET, J., and GENGOU, O., isolate the bacillus of whooping cough.
- 1906 DARLING, S. T., discovers the fungus of histoplasmosis (*Histoplasma capsulatum*).
- 1906 WASSERMANN, AUGUST VON, introduces complement-fixation test for diagnosis of syphilis.
- 1906-07 PIRQUET, CLEMENS VON, formulates idea of allergy; studies serum sickness.
- 1907 SMITH, THEOBALD, suggests use of toxin-antitoxin for immunization against diphtheria.
- 1910 RICKETTS, HOWARD T., describes rickettsiae of Rocky Mountain spotted fever and demonstrates tick transmission.
- 1910 EHRLICH, PAUL, introduces 606 or salvarsan, an arsenic-aniline compound which is an effective chemotherapeutic agent for syphilis.
- 1910-24 CARRELL, ALEXIS, investigates methods of tissue culture.
- 1911 ROUS, PEYTON, transmits fowl sarcoma by cell-free tumor filtrate.
- 1912 MCCOY, GEORGE W., and CHAPIN, CHARLES W., discover the bacillus of tularemia in (Tulare County) California ground squirrels.
- 1913 BEHRING, ÉMIL VON, uses toxin-antitoxin in prophylactic immunization against diphtheria.
- 1913 SCHICK, BÉLA, introduces skin test which detects susceptibility to diphtheria.
- 1913 DOCHEZ, A. R., and GILLESPIE, L. J., type the pneumococci.
- 1914 BARBER, M. A., introduces single-cell technique for obtaining pure cultures.
- 1914 IDO, Y., and INADA, R., establish spirochetal cause of infectious hemorrhagic jaundice (Weil's disease).
- 1915 GORDON, M. H., and MURRAY, E. G. D., distinguish serological types of meningococci.
- 1915 TWORT, F. W., observes lysis of dysentery bacilli by filtrable agent in dysentery stool; discovers bacteriophage.
- 1916 ROCHA-LIMA, H. DA, discovers rickettsiae of typhus fever.
- 1917 D'HERELLE, F., observes transmissible lysis of *Staphylococcus aureus* by filtrable agent; independently discovers bacteriophage.
- 1918 EVANS, ALICE C., establishes relationship between *Brucella melitensis* and *abortus*.
- 1921-26 KAHN, R. L., introduces serological (precipitation) test for syphilis.
- 1923 DICK, GEORGE, and DICK, GLADYS, relate hemolytic streptococcus to scarlet fever and devise skin test for susceptibility.
- 1923 RAMON, G., introduces use of toxoid in prophylactic immunization against diphtheria.
- 1924 CALMETTE, ALBERT, vaccinates children against tuberculosis with B.C.G. vaccine (Bacillus Calmette-Guérin).



# TECHNICAL METHODS OF BACTERIOLOGY

## STAINING METHODS AND FORMULAE FOR STAINS

(Not included in Chapter 9)

### Simple Stains

#### Fuchsin

##### Concentrated Alcoholic Solution

Basic fuchsin (90 per cent dye content, certified) .....	3 gm.
95 per cent ethyl alcohol .....	100 ml.

Allow to stand for 24 hours, mix thoroughly at intervals, and decant or filter, or follow directions for preparation of concentrated alcoholic solution of basic fuchsin under the Ziehl-Neelsen method page 530.

##### Fuchsin for Simple Stain and Counterstain

Concentrated alcoholic solution of basic fuchsin .....	10 ml.
Distilled water .....	90 ml.

#### Gentian Violet

Gentian violet or crystal violet (85 per cent dye content) .....	2 gm.
95 per cent ethyl alcohol .....	10 ml.

Dissolve dye by grinding in the alcohol. Mix with:

1 per cent solution of phenol .....	100 ml.
-------------------------------------	---------

#### Löffler's Methylene Blue

##### Concentrated Alcoholic Solution

Methylene blue (90 per cent dye content certified) .....	1 gm.
95 per cent ethyl alcohol .....	100 ml.
Dissolve dye in alcohol.	

##### Löffler's Methylene Blue

Concentrated alcoholic solution of methylene blue .....	30 ml.
1:10,000 solution of potassium hydroxide .....	100 ml.

#### Safranin

##### Concentrated Alcoholic Solution

Safranin .....	2.5 gm.
95 per cent ethyl alcohol .....	100 ml.

##### Safranin for Simple Stain or Counterstain

Concentrated alcohol solution .....	10 ml.
Distilled water .....	90 ml.

## Negative Stain

### *Nigrosin Solution (Dorner)*

Nigrosin	
(certified by Commission on Standardization of Biological Stains) ..	10 gm
Distilled water .....	100 ml
Boil for 30 minutes. Add as a preservative:	
Formalin .....	0.5 ml
Filter twice through double filter paper; store in small quantities.	

The procedure for negative or relief staining is given on page 126.

## Gram Stain (Hucker's Modification)

### *Ammonium Oxalate Crystal Violet Solution*

Crystal violet (85 per cent dye content certified) .....	4 gm
95 per cent ethyl alcohol .....	20 ml
1. Dissolve crystal violet in the alcohol.	
2. Dissolve ammonium oxalate in water as follows:	
Ammonium oxalate .....	0.8 gm
Distilled water .....	80 ml
3. Dilute crystal violet solution 1:10 with distilled water.	
4. Mix one part of the diluted crystal violet solution with 4 parts of ammonium oxalate solution.	

### *Gram's (or Lugol's) Iodine Solution*

Iodine crystals .....	1 gm
Potassium iodide .....	2 gm
Distilled water .....	300 ml

Dissolve the iodine and potassium iodide in the water.

A fresh solution should be prepared at least every 2 weeks.

*Safranin for Counterstain* (see page 529).

*Procedure for Gram stain* (see page 127).

## Stains for Acid-fast Bacilli

### *Ziehl-Neelsen Method*

#### *Concentrated Alcoholic Solution of Basic Fuchsin*

Basic fuchsin (certified) .....	90 gm
95 per cent ethyl alcohol .....	945 ml
1. Add 300 ml. alcohol to the 90 gm. fuchsin.	
2. Heat in water bath to boiling for 2 minutes.	
3. Allow to stand 1 minute and decant off; save the supernatant.	
4. Add a second 300 ml. portion of the alcohol to the sediment, boil and decant as before.	
5. Add the remaining 345 ml. alcohol to the sediment, boil as before and add to portions collected previously.	

#### *5 per cent Phenol*

Distilled water .....	950 ml
Phenol crystals .....	50 gm



## Carbol Fuchsin Solution for Staining Acid-fast Bacilli

Concentrated alcoholic solution of basic fuchsin .....	105 ml.
5 per cent phenol .....	895 ml.
Filter stain just before using to remove any acid-fast organisms which may be present.	

## Acid Alcohol for Decolorizing

Hydrochloric acid .....	3 ml.
95 per cent ethyl alcohol .....	97 ml.
Mix thoroughly.	

## Staining Procedure (see page 127).

## Pappenheim's Method (for differentiation between *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*).

1. Stain heat-fixed smear with hot carbol fuchsin for 2 minutes.
2. Pour off dye without washing and cover with Pappenheim's methylene blue solution which is prepared as follows:
 

Corallin (rosolic acid) .....	1 gm.
Absolute ethyl alcohol .....	100 ml.
Methylene blue added to saturation	
Glycerin .....	20 ml.

 Dissolve the corallin (rosolic acid) in the absolute alcohol. Add the glycerol and as much methylene blue as will go into solution. Keep stain tightly stoppered.
3. The above methylene blue solution is poured over the smear and drained off slowly. This procedure is repeated 4 or 5 times, and finally the preparation is washed in water. The combination of alcohol and rosolic acid decolorizes the nonpathogenic smegma bacillus and leaves the tubercle bacillus stained red. The methylene blue serves as a counterstain.

## Fluorescent Dye Method (for the identification of acid-fast organisms) \*

### Auramine O Stain

Solution A: Distilled water .....	97 ml.
Liquefied phenol .....	3 ml.
Mix thoroughly.	
Solution B: 95 per cent ethyl alcohol .....	10 ml.
Auramine O (National Aniline Co.) .....	0.1 gm.
Dissolve dye in the alcohol.	
Add Solution B to Solution A and mix.	

### Decolorizing Solution

70 per cent ethyl alcohol .....	100 ml.
Concentrated hydrochloric acid .....	0.5 ml.
Sodium chloride .....	0.5 gm.

### Staining Procedure

1. Flood heat-fixed smears with the Auramine O staining solution for 2 to 3 minutes.
2. Wash in water.

\* Richards and Miller, *Am. J. Clin. Path., Tech. Suppl. No. 5*, 11:1, 1941.

- 3. Decolorize with above solution for 2 minutes and drain slide. Flood the preparation with fresh solution and decolorize for 2 to 5 minutes.
- 4. Wash in water and dry.
- 5. Examine under a fluorescence microscope.

Stains for Metachromatic Granules

*Löffler's Methylene Blue* (see page 529)

*Stoltenberg's Stain*

Malachite green (certified) .....	1 gm.
Toluidine blue O (certified) .....	0.2 gm.
Hematoxylin (certified) .....	0.04 gm.
Glacial acetic acid .....	12 ml.
95 per cent ethyl alcohol .....	12 ml.
Distilled water .....	400 ml.

- 1. Flood heat-fixed smear with Stoltenberg's stain and allow to act for 2 to 3 minutes.
  - 2. Wash in tap water and dry.
- Metachromatic granules appear red and the body of the cell green.

*Albert's Stain* (Laybourn's Modification)

*Solution A*

Toluidine blue O (certified) .....	0.15 gm.
Malachite green (certified) .....	0.2 gm.
Glacial acetic acid .....	1 ml.
95 per cent ethyl alcohol .....	2 ml.
Distilled water .....	100 ml.

*Solution B*

Iodine crystals .....	2 gm.
Potassium iodide .....	3 gm.
Distilled water .....	300 ml.

- 1. Flood heat-fixed smears with Solution A for 3 to 5 minutes.
  - 2. Wash in tap water.
  - 3. Flood with Solution B for 1 minute.
  - 4. Wash and dry.
- Metachromatic granules stain black and the rest of the cell light green.

Capsule Stains

*Gin's Method* (Koser's modification) (see page 128)

*Hiss (Copper Sulfate) Method*

*Staining Solution*

Saturated alcoholic solution of fuchsin or gentian violet .....	5 ml.
Distilled water .....	95 ml.

- 1. Prepare smear by mixing organisms in a drop of serum.
- 2. Dry and fix by heat as usual.
- 3. Flood smear with the above stain and heat 15 to 30 seconds or until it steams.
- 4. Wash with 20 per cent aqueous copper sulfate solution and dry by blotting. (Do not wash in water.)

Capsules appear pale blue around darkly stained cell bodies.



## gella Stains

### Modified Leifson Stain (see page 129)

#### Lesares-Gil Method

##### Mordant

##### Stock Solution:

Tannic acid .....	10 gm
Aluminum chloride ( $\text{Al}_2\text{Cl}_6 \cdot 12 \text{H}_2\text{O}$ ) .....	18 gm.
Zinc chloride .....	10 gm
Rosaniline hydrochloride .....	15 gm.
60 per cent ethyl alcohol .....	40 ml

Triturate solids in 10 ml of the alcohol in a mortar.  
Add the rest of the alcohol, mixing thoroughly.

##### Dilute Solution for use:

Stock Solution of mordant .....	1 part
Distilled water .....	2 parts

##### Stain

Carbol fuchsin (see Ziehl-Neelsen Method, page 531)

##### Procedure

1. See general directions for preparation of the smear of flagellated bacteria on page 128. The importance of a properly prepared smear to a successful flagella stain cannot be over-emphasized.
2. Just before using, dilute the stock solution of the mordant as directed above and apply the filtrate directly to the slide preparation. Allow the mordant to act for 1 or 2 minutes when a precipitate and metallic sheen should form.
3. Wash gently with distilled water.
4. Flood slide with carbol fuchsin and allow it to act for 5 minutes.
5. Wash with distilled water and dry without blotting.

## re Stains

### Joeller's Method (Koser's modification) (see page 129)

#### orner's Method

##### Stains

Carbol fuchsin (see Ziehl-Neelsen Method, page 531)

Nigrosin solution (see Negative Staining, page 530)

##### Procedure

1. Make a heavy suspension by transferring organisms from an agar slant to a small tube containing 2 or 3 drops of distilled water.
2. Add 2 or 3 drops of freshly filtered carbol fuchsin to the suspension of organisms in the tube.
3. Heat the mixture in the tube in a boiling water bath for 10 to 12 minutes.
4. Mix one loopful of the stained cell suspension with one loopful of nigrosin solution on a glass slide, and quickly spread out as thin a film as possible.

The thin even smear should dry rapidly.

Spores stain red and the cell bodies appear almost colorless against the dark background of nigrosin.

## Fat Stain for Bacteria and Fungi

### *Burdon's Method* \*

#### *Sudan Black B Fat Stain*

Sudan black B (National Aniline Division, Allied Chemical and Dye Corporation) .....

0.3 gm.

70 per cent ethyl alcohol .....

100 ml.

Dissolve bulk of dye in the alcohol and then shake solution at intervals. Allow to stand overnight before using.

#### *Procedure*

1. Prepare and heat fix smear in usual way.
  2. Flood entire slide with Sudan black solution and allow to act for 5 to 15 minutes. Drying of the stain on the slide is not detrimental.
  3. Drain off excess stain.
  4. Blot slide dry.
  5. Clear the slide with xylene (c.p.).
  6. Blot slide dry.
  7. Counterstain with a 0.5 per cent aqueous solution of safranin for 5 to 10 seconds or with a 1:10 dilution of carbol fuchsin for 1 to 3 minutes. Avoid overstaining with the counterstain.
  8. Wash in water.
  9. Blot slide dry.
- Lipoid material appears blue-black or blue-gray in pink-stained cell bodies.

## INDICATORS

### Stock Solutions

1. Grind 0.1 gm. of the indicator in a mortar with the amount of N/20 sodium hydroxide listed below. To make N/20 NaOH dissolve 2 gm. NaOH in 1 liter of distilled water.
 

Brom cresol purple .....	3.7 ml. N/20 NaOH
Brom thymol blue .....	3.2 ml. "
Phenol red .....	5.7 ml. "
Cresol red .....	5.3 ml. "
2. When solution is complete, add distilled water to make 25 ml.
3. Store in tightly stoppered bottle.
4. Make up Test Solution (as described below) for use.

### Test Solutions

Dilute 1 ml. of the above stock solution with the amount of water listed below.

Brom cresol purple .....	9 ml. H <sub>2</sub> O
Brom thymol blue .....	9 ml. H <sub>2</sub> O
Phenol red .....	19 ml. H <sub>2</sub> O
Cresol red .....	19 ml. H <sub>2</sub> O

#### *pH Range*

Brom cresol purple .....	5.2 to 6.8
Brom thymol blue .....	6.0 to 7.6
Phenol red .....	6.8 to 8.4
Cresol red .....	7.2 to 8.8

\* *J. Bact.* 52:665, 1946.



## CULTURE MEDIA

Directions for the preparation of the following media are given in Chapter 11:

Nutrient broth  
Nutrient gelatin  
Nutrient agar  
Carbohydrate broth  
Carbohydrate agar  
Meat infusion broth  
Meat infusion agar  
Blood agar  
Chocolate agar

**Dextrose Proteose Peptone Broth** (may be substituted in certain instances for meat infusion broth)

Proteose peptone #3 (Difco) .....	10 gm.
Dextrose .....	0.5 gm.
Sodium chloride .....	5 gm.
Distilled water .....	1000 ml.

Heat to dissolve ingredients.

Adjust to pH 7.6.

Dispense in tubes.

Autoclave at 121° C for 15 minutes.

### Blood Broth

The addition of 2 per cent sterile defibrinated or citrated blood to meat infusion broth or dextrose proteose peptone broth provides a satisfactory medium for certainidious organisms.

### Milk with Indicator

1.6 per cent brom cresol purple (alcoholic) solution .....	1 ml.
Fresh skim milk .....	1000 ml.

Litmus, sufficient to color the milk blue, may be substituted for the brom cresol purple indicator.

Dispense in tubes, filling them one-third full.

Autoclave at 121° C for 15 minutes or sterilize by heating in streaming steam for 20 minutes on each of three successive days.

### Nitrate Broth (for the nitrite test)

Peptone .....	10 gm.
Potassium nitrate (nitrite free) .....	0.2 gm.
Distilled water .....	1000 ml.

Dissolve ingredients by heating.

Adjust reaction to pH 7.4 to 7.6

Filter through paper.

Tube in 3 to 5 ml. amounts.

Autoclave at 121° C for 15 minutes.

***Tryptone Broth*** (for indole production)

Tryptone (Bacto) .....	5 gm.
Sodium chloride .....	5 gm.
Distilled water .....	1000 ml.

1. Heat to dissolve ingredients.
2. Adjust to pH 7.4 to 7.6.
3. Filter through paper if necessary.
4. Tube and autoclave at 121° C for 15 minutes.

***Semisolid Agar***

Beef infusion broth (or any other suitable broth base) .....	1000 ml.
Agar .....	1.5 gm.

1. Heat mixture to dissolve agar.
2. If necessary add distilled water to restore original volume.
3. Adjust to pH 7.4 to 7.6
4. Tube and autoclave at 121° C for 15 minutes.

***Carbohydrate Semisolid Agar*** (for fermentation studies on *Neisseria*)

Semisolid (beef infusion) agar, pH 7.6 .....	1000 ml.
Brom thymol blue, 0.2 per cent alcoholic solution .....	5 ml.
Carbohydrate, sterile 5 per cent solution.	

1. Mix the indicator with the agar.
2. Tube in 5 ml. amounts.
3. Autoclave at 121° C for 15 minutes. Store until needed.
4. Before use, heat tubes in a water bath to melt the agar, and add to each tube 0.5 ml. of a sterile 5 per cent solution of the desired carbohydrate. Carbohydrate solutions should be sterilized by filtration or by exposing to live steam in an Arnold sterilizer for 20 minutes on each of three successive days.

***Bordet-Gengou (Potato Blood) Agar*** (for *Hemophilus pertussis*)***Potato glycerin agar base***

Peeled, sliced or ground potatoes .....	500 gm.
Glycerin .....	40 ml.
Distilled water .....	1000 ml.

1. Boil or steam the above mixture until potatoes are soft.
2. Make up to the original volume by adding distilled water.
3. Strain through 3 or 4 thicknesses of gauze.
4. To each 500 ml. of the potato glycerin fluid add:
 

0.75 per cent sodium chloride solution .....	1500 ml.
Agar .....	50 gm.
5. Heat gently to dissolve the agar.
6. Restore to volume by adding distilled water.
7. Dispense into flasks in 100 ml. (or multiples of 100 ml.) amounts.
8. Autoclave at 121° C for 25 minutes.

***Complete medium***

1. To each 100 ml. of melted and cooled (45° C) potato glycerin agar base add 20 ml. fresh defibrinated or citrated sheep, rabbit or human blood.



Mix blood with agar base and pour into plates. Medium in plates should be moist, smooth and cherry red in color when used not more than 72 hours after pouring.

**Line Tellurite Blood Agar (Frobisher) (for *Corynebacterium diphtheriae*)**

Add 5 mg. cystine (dry powder) to 100 ml. of melted and cooled (55° C.) meat infusion agar. Hold agar at 50° C.

Add 5 ml. of sterile defibrinated or citrated sheep or rabbit blood to the agar.

Add 15 ml. of sterile 0.3 per cent potassium tellurite solution to the agar. This will give a final concentration of 0.0375 per cent potassium tellurite in the medium. The optimal amount of potassium tellurite to produce luxuriant growth of typical black colonies of *C. diphtheriae* and to inhibit most contaminating organisms in a specimen can be determined only by testing varying amounts of a particular lot of potassium tellurite. A range of 0.0025 per cent to 0.125 per cent final concentration is suggested for testing new lots of this substance.

Keep medium thoroughly mixed by occasional agitation while pouring plates.

Pour about 12 to 15 ml. of medium into each plate.

Test sterility by incubating 24 hours before using.

**er (Serum) Medium (for *Corynebacterium diphtheriae*)**

Fresh blood serum .....	3 parts
1 per cent dextrose meat infusion broth .....	1 part

One per cent dextrose beef extract broth may be substituted for the infusion broth.

Dispense mixture into wide-mouthed tubes in 3 to 5 ml. amounts.

Place 8 to 10 tubes (2 or 3 rows) in a one gallon, wide-mouthed, glass jar fitted with a metal screw cap which has been punctured to leave a hole about 3 mm. in diameter. A ring of rubber tubing placed half way up the jar supports the tubes at an appropriate slant when the jar is placed on its side in the autoclave. Jar cap must be screwed on securely.

Coagulate the medium as follows:

- Close all ports and the door of the autoclave before turning on the steam.
- Raise the pressure gradually to 15 lb. pressure with no escape of air.
- Hold at this pressure for 10 minutes.

Sterilize the medium as follows:

- At end of coagulation time open the air outlet valve very slightly and regulate the steam supply so that the air-steam mixture is replaced by live steam without a change in the pressure. A constant pressure of 15 lb. must be maintained.
- Sterilize by steam under 15 lb. pressure (121° C) for 20 minutes.
- At end of sterilization time, turn off steam, close tightly all ports and safety valve, and allow autoclave to cool slowly. Do not open autoclave until pressure is zero.

Satisfactory medium must have a smooth, even surface.

**od Glucose Cystine Agar (Francis) (for *Pasteurella tularensis*)**

To each 100 ml. of fresh, sterile, beef infusion agar add:

Cystine (pulverized) .....	0.1 gm.
Glucose .....	1 gm.

Expose the mixture to streaming steam long enough to melt the agar and to sterilize the cystine and glucose.

3. Cool to 50° C and add 5 to 8 per cent sterile defibrinated or whole rabbit blood.
4. Heat the medium in a water bath at 60° C for 2 hours.
5. Dispense medium from flask or a sterile funnel into plates or tubes. Slant medium in tubes.
6. Incubate plates and tubes for 24 hours to check sterility.

The surface of the medium should be relatively free from water of condensation before using.

### **Liver Infusion Agar (for *Brucella*)**

#### *Liver infusion*

Ground raw liver .....	500 gm
Distilled water .....	1000 ml

1. Infuse the liver in the water overnight in the refrigerator.
2. Skim off the fat.
3. Heat to 50° C and hold at this temperature for 1 hour.
4. Boil for ½ hour.
5. Remove coagulum.
6. Strain to remove particles.
7. Make up to original volume with distilled water.

#### *Liver infusion agar*

Liver infusion .....	500 ml
Peptone .....	10 gm
Sodium chloride .....	5 gm
Agar .....	20 gm
Distilled water .....	500 ml

1. Heat to dissolve ingredients.
2. Cool to 60° C and adjust to pH 7.0.
3. Boil or expose to live steam for 20 minutes.
4. Decant off clear agar or filter through coarse strainer.
5. Adjust reaction to pH 7.0. (Final reaction will be about pH 6.6.)
6. Dispense to tubes or flasks and autoclave at 121° C for 30 minutes.
7. Incubate for 24 hours to test sterility.

### **Liver Infusion Agar with Thionin or Basic Fuchsin (for differentiation of *Brucella* species)**

Liver infusion agar (pH 6.6), sterile ..... 500 ml

plus

Thionin, 1 per cent sterile aqueous solution ..... 1 ml

or

Basic fuchsin, 1 per cent sterile aqueous solution ..... 2 ml

Sterile dye solutions are prepared by weighing the dry dye in a sterile tube, adding the proper amount of distilled water and heating in flowing steam for 20 minutes. The hot dye solution should be shaken thoroughly before adding to the agar base.

### **Petragnani Medium (for *Mycobacterium tuberculosis*)**

Potato, peeled and sliced thin .....	75 gm
Fresh skim milk .....	150 ml
Potato flour .....	6 gm



Peptone .....	10 gm.
Eggs, whole .....	4
Egg yolk .....	1
Glycerin .....	12 ml.
Malachite green (certified), 2 per cent aqueous solution .....	12 ml.

1. Cook potato, potato flour, peptone and milk in a double boiler for 2 hours. Stir continuously until mixture becomes sticky and then stir occasionally.
2. Beat the eggs and egg yolk with the glycerin and malachite green solution until thoroughly mixed.
3. Cool potato mixture to 50° C and add the egg mixture. Mix well.
4. Filter through gauze.
5. Dispense into large-mouthed test tubes in 3 to 5 ml. amounts.
6. Coagulate medium in slant position and sterilize according to the directions for coagulation and sterilization of Löffler's (blood serum) medium.

## Media for Differentiation of Intestinal Bacteria

### *Bismuth Sulfite Agar* (Wilson and Blair as modified by Hajna)

#### *Agar base*

Agar .....	30 gm.
Beef extract .....	5 gm.
Peptone .....	10 gm.
Glucose .....	5 gm.
Distilled water .....	1000 ml.

1. Heat to dissolve ingredients in the water.
2. Restore original volume with distilled water.

#### *Bismuth Sulfite Mixture*

1. Sodium sulfite, anhydrous ( $\text{Na}_2\text{SO}_3$ ) ..... 80 gm.  
Distilled water ..... 400 ml.  
Dissolve sodium sulfite in the water by heat and agitation.
2. Bismuth citrate (Merck USP VIII) ..... 24 gm.  
Distilled water ..... 200 ml.  
Concentrated ammonia (Sp. Gr. 0.897) ..... 12 ml.  
Mix the bismuth citrate in 40 ml. of the water to form a paste. When a solution is formed add the rest of the water.
3. Combine the sodium sulfite and bismuth citrate solutions, and add to the mixture:  
Dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) ..... 42 gm.
4. Make up the following solution:  
Ferrous sulfate crystals ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) ..... 4 gm.  
Distilled water ..... 40 ml.  
Concentrated hydrochloric acid ..... 2 drops (0.075 ml.)
5. When sulfate crystals are dissolved in water containing the hydrochloric acid, immediately add 40 ml. of the sulfate solution to the mixture of 1, 2, and 3 above, mixing thoroughly.
6. Boil mixture gently for 2 minutes, when it should be slate gray.

#### *Complete Medium*

Bismuth sulfite mixture .....	70 ml.
Brilliant green (certified), 1 per cent aqueous solution .....	4 ml.
Agar base, hot melted .....	1000 ml.

1. Mix well.
2. Just before pouring plates, autoclave at  $121^{\circ}\text{C}$  for not more than 10 minutes. The final pH should be approximately pH 7.8.
3. Pour 20 ml. amounts into porous top plates or regular plates from which the glass cover is partially removed to avoid wetting of the agar surface by the water of condensation. Keep away from direct sunlight.
4. Allow plates to remain at room temperature for 1 to 2 hours before using or storing. Plates should be stored in the refrigerator for not longer than 4 days.

Gram-positive and coliform organisms as well as many members of the dysentery group are inhibited by this medium. *Salmonella typhosa* and *S. schottmülleri* grow luxuriantly and produce black colonies. Many other *Salmonella* such as *S. paratyphi* and *S. typhimurium* produce green colonies.

### ***Desoxycholate Citrate Agar* (Leifson)**

#### *Meat Infusion Base*

Meat, fresh lean ground .....	400 gm.
Distilled water .....	1200 ml.
Normal hydrochloric acid .....	2 ml.

1. Infuse meat in water at room temperature for 1 hour.
2. Add hydrochloric acid and mix.
3. Boil for 5 minutes.
4. Strain through gauze and filter through paper until clear and fat is removed.
5. Adjust to pH 8.0 by addition of normal sodium hydroxide.
6. Boil for 10 minutes.
7. Filter through paper.
8. Make up to original volume by adding distilled water.
9. Adjust to pH 7.4.
10. Use as base of agar medium as directed below or autoclave in flasks at  $121^{\circ}\text{C}$  for 30 minutes and store in refrigerator until needed.

#### *Complete Medium*

Meat infusion base .....	100 ml.
Peptone .....	1 gm.
Agar .....	2 gm.
Lactose .....	1 gm.
Sodium citrate .....	2 gm.
Ferric ammonium citrate .....	0.2 gm.
Sodium desoxycholate .....	0.5 gm.
Neutral red, 0.1 per cent aqueous solution .....	2 ml.

1. Boil agar and peptone in the broth base for 3 minutes.
2. Restore 100 ml. volume by adding distilled water.
3. Add and dissolve the lactose, both citrate salts and sodium desoxycholate.
4. Adjust to pH 7.3 to 7.5.
5. Add the neutral red solution.
6. Expose to streaming steam (Arnold sterilizer) for 15 minutes. Excess heat is detrimental to this medium.
7. Pour 15 ml. amounts into porous-top Petri plates or regular plates and treat as directed for bismuth sulfite agar.

Coliform bacilli are considerably inhibited by this medium; those that grow produce red colonies. Colonies of *Salmonella* and *Shigella* bacilli are colorless.



**Eosin-Methylene Blue Agar (Levine)****Beef Extract Agar Base**

Agar .....	20 gm.
Beef extract .....	5 gm.
Peptone .....	15 gm.
Dibasic potassium phosphate ( $K_2HPO_4$ ) .....	2 gm.
Distilled water .....	1000 ml.

1. Boil to dissolve ingredients.
2. Restore original volume by adding distilled water.
3. Filter through cotton.
4. Autoclave in flasks at  $121^\circ C$  for 20 minutes.

**Complete Medium**

Agar base, sterile .....	100 ml.
Lactose, sterile 20 per cent solution .....	5 ml.
Eosin Y, 2 per cent aqueous solution .....	2 ml.
Methylene blue, 0.5 per cent aqueous solution .....	2 ml.

Mix thoroughly and pour into plates as described for bismuth sulfite agar.

*Escherichia coli* colonies are dark colored with a metallic sheen and almost black centers. Colonies of *A. aerogenes* are colored but less so than those of the coliform bacilli. *Salmonella* and *Shigella* colonies are colorless.

**Endo Agar (Robinson and Rettger Modification)**

Beef extract agar base (substitute 25 gm. agar in the formula given under eosin-methylene blue agar) .....	1000 ml.
Sodium carbonate, 10 per cent aqueous solution .....	10 ml.
Lactose .....	10 gm.
Sodium bisulfite, 10 per cent aqueous solution .....	10 ml.
Basic fuchsin, saturated (10 per cent) alcoholic solution, amount determined by standardization only .....	0.5 to 3.0 ml.

1. Add the carbonate solution to the agar base. Mix well.
2. Adjust to pH 7.6 to 7.8.
3. Steam for 10 minutes.
4. Add lactose, fuchsin and sodium sulfite. Mix well.
5. Dispense into flasks.
6. Autoclave at  $115^\circ$  to  $116^\circ C$  for 20 minutes.
7. Pour 15 to 20 ml. amounts into plates as described for bismuth sulfite agar.
8. Store medium in refrigerator in the dark. Use plates within 4 or 5 days after pouring.

On this medium *Esch. coli* produces deep red colonies with a metallic sheen. Colonies of *A. aerogenes* are similar to those of the coliform bacilli, but are less intensely colored. *Salmonella* and *Shigella* colonies are colorless.

**MacConkey Bile Salt Agar**

Peptone .....	20 gm.
Lactose .....	10 gm.
Sodium chloride .....	5 gm.
Bile salts No. 3 (Bacto) .....	5 gm.
Agar .....	15 gm.
Distilled water .....	1000 ml.

Neutral red, 1 per cent aqueous solution (amount may vary with different dye lots) ..... 5 ml

- 1. Dissolve dry ingredients in the water by heating in a water bath or autoclaving.
- 2. Make up to original volume by adding distilled water.
- 3. Adjust to pH 7.4.
- 4. Add the neutral red solution.
- 5. Autoclave at 121 ° C for 20 minutes.
- 6. Pour 15 to 20 ml. amounts into plates and dry surface by allowing plates to stand for 2 hours with tops partially removed. Use plates within 4 or 5 days after pouring.

Coliform bacilli produce red colonies surrounded by a zone of precipitated bile salts. *Salmonella* and *Shigella* organisms produce transparent, colorless colonies.

**S S Agar** (for *Salmonella* and *Shigella* organisms)

*Bile salt agar base*

Beef extract .....	5 gm
Proteose peptone .....	5 gm
Bile salts No. 3 (Difco) .....	8.5 gm
Agar .....	17 gm
Distilled water .....	1000 ml.

- 1. Dissolve dry ingredients in the water by heating in water bath or autoclave.
- 2. Restore original volume by adding distilled water.
- 3. Dispense into flasks and autoclave at 121 ° C for 30 minutes.

*Complete Medium*

Bile salt agar base .....	1000 ml.
Lactose .....	10 gm.
Sodium citrate .....	8.5 gm.
Sodium thiosulfate .....	8.5 gm.
Ferric citrate .....	1 gm.
Neutral red, 1 per cent aqueous solution .....	2.5 ml.
Brilliant green, 0.1 per cent aqueous solution .....	0.33 ml.

- 1. Add the dry ingredients to the hot melted agar base and mix thoroughly to dissolve.
- 2. Adjust to pH 7.0.
- 3. Add neutral red and brilliant green solutions. Mix well. Do not autoclave.
- 4. Pour 15 to 20 ml. amounts into plates, and allow plates to stand with tops partially removed for 2 hours or until surface of medium is quite dry.

Growth of coliform bacilli is considerably inhibited on this medium; if growth occurs colonies are red- or pink-centered. *Salmonella* and *Shigella* colonies are colorless.

**Tetrathionate Enrichment Medium** (to inhibit coliform organisms and facilitate the primary isolation of *Salmonella*, including the typhoid bacillus, from specimens)

*Tetrathionate broth base*

Proteose peptone No. 3 (Difco) .....	5 gm.
Bile salts No. 3 (Bacto) .....	1 gm.
Calcium carbonate .....	10 gm.
Sodium thiosulfate .....	30 gm.
Brilliant green, 0.1 per cent aqueous solution .....	11 ml.
Distilled water .....	1000 ml.



1. Dissolve peptone and bile salts in the water.
2. Dispense into flasks in 500 ml. amounts.
3. Add 5 gm. calcium carbonate to each flask.
4. Autoclave at 121° C for 20 minutes. Store.
5. When needed add 15 gm. sodium thiosulfate and 5.5 ml. brilliant green solution to each flask, and mix thoroughly.
6. Dispense aseptically into tubes in 10 ml. amounts. Keep suspension of calcium carbonate uniform by agitation.

## Iodine solution

Iodine crystals .....	30 gm.
Potassium iodide .....	25 gm.
Distilled water .....	100 ml.

Dissolve dry ingredients in the water.

## Complete Medium

Just before inoculating, add 0.2 ml. of the iodine solution to each tube of tetrathionate broth base.

Add 1 to 3 gm. feces or other specimen to one tube of medium and mix thoroughly. After 12 to 24 hours' incubation streak broth culture out on MacConkey, SS, and bismuth sulfite agar plates, and incubate for production of typical colonies.

## Selenite-F Enrichment Medium (Leifson) (for the primary isolation of *Salmonella* including typhoid bacilli)

Sodium hydrogen selenite (anhydrous) .....	4 gm.
Peptone .....	5 gm.
Lactose .....	4 gm.
Sodium phosphates * (anhydrous) .....	10 gm.
Distilled water .....	1000 ml.

1. Dissolve ingredients in the water.
2. Adjust pH as recommended above.
3. Tube in 10 ml. amounts (to a depth of 2 inches or more).
4. Sterilize by exposure to streaming steam for 30 minutes. The medium must not be autoclaved.

Emulsify 1 to 2 gm. of feces in one tube of medium. Incubate at 37° C for 18 to 24 hours, and streak the culture on desoxycholate citrate agar, SS agar, or other suitable plate media.

## Russell Double Sugar Agar (for detection of dextrose-acid, dextrose-acid-and-gas, lactose-acid, and lactose-acid-and-gas-forming organisms)

Nutrient agar (pH 7.2 to 7.6) .....	1000 ml.
Lactose .....	10 gm.
Dextrose .....	0.5 gm.
Phenol red, 0.02 per cent aqueous solution .....	30 ml.

\* A combination of dibasic sodium phosphate (anhydrous) and monobasic sodium phosphate is used which will result in a medium with a final pH of about 7.0. The proportions vary with the kind of peptone and lot of selenite employed: Leifson used 3 parts of dibasic sodium phosphate to 1 part monobasic sodium phosphate with sodium hydrogen selenite prepared by the Baltimore Biological Laboratory and Wilson's CB peptone.

1. Dissolve the sugars in the hot, melted nutrient agar and add the indicator. Mix well.
2. Dispense in 3 to 5 ml. amounts in tubes.
3. Autoclave at 121 ° C for 15 minutes.
4. Cool tubes in slanting position designed to result in a short slant above deep butt.

**Lead Acetate Agar** (for detection of hydrogen sulfide production)

- |                                |         |
|--------------------------------|---------|
| Peptone .....                  | 15 gr   |
| Proteose peptone (Difco) ..... | 5 gr    |
| Dextrose .....                 | 1 gr    |
| Lead acetate, basic .....      | 0.2 gr  |
| Agar .....                     | 15 gr   |
| Distilled water .....          | 1000 ml |
1. Dissolve peptone, proteose peptone, dextrose and agar in the water by gentle heating.
  2. Restore original volume by adding distilled water.
  3. Adjust to pH 7.2.
  4. Add 10 ml. of a 0.2 per cent aqueous solution of basic lead acetate.
  5. Tube and sterilize by autoclaving at 121 ° C for 15 minutes.
  6. Allow medium to harden in tubes in upright position.

Inoculate by stabbing into the medium at several points next to the side of the tube.

**Koser's Citrate Medium** (for differentiation of *Escherichia coli* and *Aerobacter aerogenes*)

- |                                                                                  |          |
|----------------------------------------------------------------------------------|----------|
| Sodium ammonium phosphate (4 H <sub>2</sub> O) .....                             | 1.5 gm   |
| Monobasic potassium phosphate (KH <sub>2</sub> PO <sub>4</sub> ) anhydrous ..... | 1 gm     |
| Magnesium sulfate, anhydrous .....                                               | 0.2 gm   |
| Sodium citrate (2 H <sub>2</sub> O) .....                                        | 3 gm     |
| Distilled water .....                                                            | 1000 ml. |
1. Dissolve ingredients in the water.
  2. Adjust to pH 6.7 to 6.9.
  3. Tube and sterilize by autoclaving at 121 ° C for 15 minutes.

*A. aerogenes* can utilize the citrate salt as the sole source of carbon in the medium, whereas *Esch. coli* cannot. Therefore *A. aerogenes* grows in the medium and *Esch. coli* does not.

**Simmons Citrate Agar** (for differentiation between *Escherichia coli* and *Aerobacter aerogenes*, and between certain members of the *Salmonella*-typhoid-dysentery group)

- |                                                       |          |
|-------------------------------------------------------|----------|
| Monobasic ammonium phosphate .....                    | 1 gm.    |
| Dibasic potassium phosphate .....                     | 1 gm.    |
| Magnesium sulfate .....                               | 0.2 gm.  |
| Sodium citrate .....                                  | 2 gm.    |
| Sodium chloride .....                                 | 5 gm.    |
| Agar .....                                            | 20 gm.   |
| Distilled water .....                                 | 1000 ml. |
| Brom thymol blue, 0.4 per cent aqueous solution ..... | 20 ml.   |



1. Dissolve salts and agar in the water by heating.
2. Restore original volume by adding distilled water.
3. Adjust reaction to pH 6.8 to 7.0.
4. Add brom thymol blue solution. Mix well.
5. Dispense in 3 to 5 ml. amounts in tubes and autoclave at 121° C for 15 minutes.
6. Cool tubes in a slanting position.

*Aerobacter aerogenes*, *Salmonella schottmülleri*, *Salmonella enteritidis*, and *Salmonella typhimurium* can utilize the citrate salt and grow on this medium, turning it a deep blue color due to the production of alkali. *Salmonella paratyphi*, the typhoid bacillus and dysentery bacilli cannot grow on this medium. *E. coli* grows poorly, with no color change in the medium, or, usually, does not grow at all.

**M R - V P Medium** (for differentiating between *Escherichia coli* and *Aerobacter aerogenes* by the methyl red test and the Voges-Proskauer test)

Peptone .....	7 gm.
Dextrose .....	5 gm.
Dibasic potassium phosphate .....	5 gm.
Distilled water .....	1000 ml.

1. Dissolve dry ingredients in the water.
2. Adjust reaction to pH 7.2.
3. Dispense in 5 ml. amounts in tubes.
4. Autoclave at 121° C for 15 minutes.

## Media for Anaerobes

### Glucose Brain Broth (Rosenow)

Meat infusion .....	1000 ml.
Peptone .....	5 gm.
Sodium chloride .....	8 gm.
Glucose .....	2 gm.
Calf brain cut into pieces 1 cm. square	
Marble, crushed	

1. Dissolve peptone, salt and glucose in meat infusion by gentle heating.
2. Adjust reaction to pH 7.6 to 7.8.
3. Dispense into tall tubes (about 1.5 cm. diameter) until column of broth is 12 cm. deep.
4. To each tube add 2 or 3 small pieces of crushed marble and 3 pieces of calf brain. Wet pieces of brain to avoid sticking to sides of tube.
5. Autoclave at 121° C for 20 minutes. Store in refrigerator.
6. Heat tubes in boiling water bath for 20 minutes to drive off dissolved oxygen and cool quickly just before inoculating.

### Cooked Meat Medium

Ground lean meat .....	500 gm.
Distilled water .....	1000 ml.
Peptone .....	20 gm.
Sodium chloride .....	5 gm.
Dextrose .....	1 gm.

1. Steep the meat in the water overnight in the refrigerator.
2. Remove scum of fat.
3. Strain through muslin cloth. After expressing as much liquid from it as possible save the meat.
4. Restore the infusion to original volume by adding distilled water.
5. Add the peptone, salt and dextrose and dissolve by mixing and gentle heat.
6. Adjust to pH 7.8 to 8.0.
7. Fill tall tubes (about 1.5 cm. diameter) with a 6 to 7 cm. column of the broth.
8. To each tube add enough of the finely divided cooked meat to occupy about one half the column of liquid, or to make the final column of medium 10 to 12 cm. deep.
9. Autoclave at 121° C for 20 minutes. Store in refrigerator.
10. Boil tubes in water bath for 20 minutes to drive off dissolved oxygen and cool quickly just before inoculating.

### *Thioglycollate Broth* (Brewer)

Peptone, thio .....	10 g
Glucose .....	10 g
Sodium chloride .....	5 g
Dibasic potassium phosphate .....	2 g
Sodium thioglycollate .....	1 g
Agar .....	0.5 g
Methylene blue, 1:5000 aqueous solution .....	10 ml
Meat infusion .....	750 ml
Distilled water to make final volume .....	1000 ml

1. Dissolve ingredients in the meat infusion by heating.
2. Add distilled water to make up to 1000 ml.
3. Adjust to pH 7.4 to 7.6.
4. Add methylene blue solution. The reduced medium is amber colored; oxidation is indicated by a greenish color starting at the top of the column of medium.
5. Fill tubes to a height of about 7 cm.
6. Autoclave at 121° C for 20 minutes.
7. Store at **room temperature**.
8. When more than a centimeter of the medium has been oxidized, as indicated by a greenish color, the medium must be boiled for a few minutes and cooled before use.

### Medium for Aciduric Bacteria (*Lactobacilli*)

#### *Tomato Juice Agar* (Kulp and White)

Tomato juice from canned tomatoes, filtered through paper .....	400 ml
Peptonized milk (Difco) .....	10 g
Peptone .....	10 g
Agar .....	11 g
Distilled water .....	600 ml

1. Dissolve peptonized milk and peptone in the tomato juice by gentle heating. Avoid unnecessary heat.
2. Adjust reaction of tomato juice mixture to pH 6.0 to 6.2.
3. Dissolve the agar in the water by boiling or steaming. Restore to original volume.



4. Combine hot tomato juice mixture with hot agar-water.
5. Filter through a thin layer of absorbent cotton.
6. Dispense into flasks or tubes and sterilize by autoclaving at 121° C; hold the tubes at this temperature for 8 minutes and the flasks for a little longer. Remove medium from autoclave as soon as pressure is nil. The final pH should be 6.0 to 6.2.

## Medium for Detection of Lipolytic Activity

### Nile Blue Sulfate Fat Agar

#### Fat emulsion

Cottonseed oil .....	3 ml.
Agar .....	0.5 gm.
Distilled water .....	100 ml.

1. Mix ingredients and sterilize by autoclaving at 121° C for 20 minutes.
2. Shake well before using in complete medium.

#### Complete medium

Nutrient agar, sterile, melted, cooled (50° C) .....	100 ml.
Fat emulsion, sterile .....	4 ml.
Nile blue sulfate, sterile 0.2 per cent aqueous solution .....	5 ml.

Shake well and pour plates.

Nile blue sulfate stains fat pink and the organic acids resulting from digestion of fat are colored dark blue. Colonies surrounded by dark blue zones contain lipolytic bacteria.

## Media for Yeasts and Molds

### Sabouraud Glucose Agar

Peptone .....	10 gm.
Glucose .....	40 gm.
Agar .....	20 gm.
Distilled water .....	1000 ml.

1. Dissolve agar in water by boiling.
  2. Dissolve the peptone in the hot agar solution by stirring.
  3. Dissolve the dextrose in hot peptone agar by stirring.
  4. Restore original volume by adding distilled water.
  5. Adjust reaction to pH 5.8 to 6.0.
  6. Dispense into flasks or tubes.
  7. Autoclave at 121° C for 30 minutes.
- Final reaction should be pH 5.2 to 5.6.

### Henrici Glucose Tartaric Acid Agar

#### Glucose tartaric acid solution

Glucose .....	25 gm.
Tartaric acid, crystals .....	2.5 gm.
Distilled water .....	50 ml.

1. Dissolve ingredients in water.
2. Dispense into flask or tubes.
3. Autoclave at 121° C for 15 minutes.

*Complete medium*

Nutrient agar, sterile melted, pH 7.4 .....	100
Glucose tartaric acid solution, sterile .....	10

Mix thoroughly and pour plates.

For slants, add 1 ml. of the sterile glucose tartaric acid solution to each 1 tube of sterile melted nutrient agar. Mix and allow medium to harden in position.

Final reaction of medium is about pH 3.8.

**Corn Meal Agar** (especially valuable for development of mycelium chlamydospores of *Candida albicans*)

Corn meal, yellow .....	40
Agar .....	20
Distilled water .....	1000

1. Add corn meal to 500 ml. of the water and hold at 65° C for 1 hour.
2. Filter through paper. Restore volume to 500 ml. with distilled water.
3. Dissolve agar in 500 ml. water by boiling. Restore to original volume.
4. Combine corn meal filtrate and agar. Mix thoroughly. No adjustment of is necessary.
5. Dispense in tubes or small flasks, and autoclave at 121° C for 20 minutes.

If dehydrated medium is used for observation of chlamydospore production by *Candida*, one must be sure that it contains no glucose.

*Littman Oxgall Agar*

Dextrose .....	1 g
Peptone .....	1 g
Oxgall (dehydrated) .....	1.5 g
Agar .....	2 g
Crystal violet .....	0.001 g
Distilled water .....	100 ml

1. Dissolve ingredients in water.
2. Do not adjust reaction of medium.
3. Dispense in tubes or flask.
4. Sterilize at 10-12 lb. pressure at 115°-117° C for 15 minutes.
5. Cool agar to 46° C and add streptomycin sulfate to provide a final concentration of 30 µgm. per milliliter of medium.

**TESTS FOR BIOCHEMICAL ACTIVITIES****Ammonia Production***Test Using Nessler's Reagent*

1. Grow organism in a medium containing proteins or peptone at 37° C for 5 days.
2. Heat culture.
3. Dip a piece of filter paper into Nessler's solution and expose the paper to vapor of the heated culture.

A positive test is indicated by the paper's turning red, brown or black depending on the amount of ammonia present in the culture.



## Thomas Method

### Reagents

Phenol, 4 per cent aqueous solution.

Sodium hypochlorite solution containing about 1 per cent available chlorine.

### Procedure

1. Grow organism in a peptone medium at 37° C for 5 days.
2. Dilute 0.2 ml. of the culture to a volume of 8 ml. by adding distilled water.
3. Add 1 ml. of the phenol solution and 1 ml. of the hypochlorite solution, and mix thoroughly.

A blue color is positive for ammonia. In an amber colored medium a green or greenish blue is a positive test.

## Indole Production

### Ehrlich-Böhme Method

#### Reagents

##### Solution A.

<i>p</i> -dimethylaminobenzaldehyde .....	4 gm.
Ethyl alcohol, 95 per cent .....	380 ml.
Hydrochloric acid, concentrated .....	80 ml.

Add the HCl to a solution of the first two ingredients.

##### Solution B.

Potassium persulfate, 1 per cent aqueous solution.

### Procedure

1. Grow organisms in tryptone broth or another tryptophane-rich medium at 37° C for 4 or 5 days.
2. To approximately 5 ml. of the culture add 1 ml. of Solution A and 1 ml. of Solution B by running the reagents down the side of the tube to form a layer over the culture medium.

A red color, which may take a few minutes to develop, indicates the presence of indole. The use of Solution B intensifies the reaction but is optional.

## Production of Nitrite from Nitrate (nitrate reduction test)

### Reagents

#### Solution A.

Sulfanilic acid .....	0.8 gm.
Acetic acid, 5/N (1 part glacial acetic acid to 2.5 parts distilled water) .....	100 ml.

#### Solution B.

$\alpha$ -naphthylamine .....	0.5 gm.
Acetic acid, 5/N .....	100 ml.

### Procedure

1. Grow organism in nitrate broth at 37° C for 3 to 5 days.
2. Add a few drops of Solution A and the same amount of Solution B to the culture.

A pink to red color indicates the presence of nitrite (a positive nitrate reduction test).

### Methyl Red (MR) Test

1. Grow organism in MR-VP medium (buffered glucose-peptone broth) at 37°C for 3 to 5 days.
2. Add 5 drops of a 0.04 per cent solution of methyl red indicator to approximately 5 ml. of the culture.

A red color indicates acid or MR positive; a yellow color is MR negative. *Escherichia coli* cultures give a positive MR test; *Aerobacter aerogenes* cultures, a negative MR test.

### Voges-Proskauer (VP) Test (O'Meara's Modification) (for acetyl-methyl carbinol)

1. Grow organism in MR-VP medium (buffered glucose-peptone broth) at 37°C for 2 days.
2. Add to the culture a small amount of creatine (amount picked up on the end of an applicator stick) and 5 ml. of 40 per cent sodium hydroxide.
3. Shake tube for 2 to 5 minutes.

The appearance of a pink color within a few minutes to an hour or longer indicates the presence of acetyl-methyl carbinol (a positive VP test). *Aerobacter aerogenes* gives a positive VP test; *Escherichia coli*, a negative VP test.

### Utilization of Citrate (as a source of carbon)

See Koser citrate medium and Simmons citrate medium.

### Hydrogen Sulfide Production

Brown or black discoloration of lead acetate medium is a positive test for hydrogen sulfide production.

### Oxidase Test (for detection of *Neisseria* colonies)

#### Reagent

Tetramethyl <i>p</i> -phenylenediamine hydrochloride .....	1
Distilled water .....	100

Dissolve the dye in the water. A fresh solution should be prepared weekly.

#### Procedure

1. Flood the surface of the plate with from 1 to 2 ml. of the reagent, and pour off excess solution.
2. Colonies of bacteria (including *Neisseria*) which form indophenol oxidase turn purple when exposed to the reagent (positive oxidase test).
3. Organisms from treated colonies are alive and may be subcultured if transferred within half an hour.

### Miscellaneous Methods

#### *Lactophenol Mount* (for microscopic examination of filamentous fungi)

##### *Lactophenol solution*

Phenol crystals .....	20
Lactic acid .....	20
Glycerol .....	40
Distilled water .....	20

Mix well. Dissolve by heating gently in a water bath. Cotton Blue (0.05 g) may be added to stain the fungi.



## Preparation of mount

A bit of the mold colony is removed from an agar culture by cutting around and slightly below the surface growth with a pair of sharp pointed forceps. The piece is then lifted from the plate (using the forceps as a spatula) and is placed in a drop of the lactophenol solution taking care not to disturb the aerial hyphae. Enough lactophenol is added to cover the specimen completely and to prevent the cover slip from exerting too much pressure on it. The cover slip is applied gently, contacting first one edge with the fluid and lowering gradually over the mount.

## Methylene Blue Reduction Method (Reductase Test) (for relative number of bacteria in milk)

1. Prepare a methylene blue solution by adding 1 standard tablet to 200 ml. water. Standard methylene blue tablets prepared by the National Aniline Company may be purchased from laboratory supply houses.
2. Place 10 ml. milk in a sterile test tube and add 1 ml. of the methylene blue solution. Mix thoroughly.
3. Immediately place tube in a 37° C water bath and note the time of this, the beginning of the test.
4. Observe tube frequently. Note the time when the blue color entirely disappears, leaving the milk white.
5. Interpret the quality of the milk from the following or another, similar table.

QUALITY OF MILK	TIME FOR DECOLORIZATION	APPROXIMATE NUMBER OF COLONIES PER ML. ON AGAR PLATE
Good	Not decolorized in 5½ hours	Less than 500,000
Fair	2 to 5½ hours	500,000 to 4,000,000
Unsatisfactory	20 minutes to 2 hours	4,000,000 to 20,000,000
Very unsatisfactory	20 minutes or less	Over 20,000,000

## Breed Method (for direct microscopic count of bacteria in milk)

1. Place clean slide over a guide which is marked off into 1 cm. squares.
2. With a special (Breed) capillary pipette remove 0.01 ml. milk from a well-shaken sample and deposit on the slide over the center of a 1 cm. square.
3. Spread the milk evenly over the entire square with a clean, straight needle.
4. Dry the film quickly in a warm (not hot) place.
5. Remove fat from the film by soaking in xylene for 1 to 2 minutes. Drain and air-dry slide in an upright position.
6. Fix by placing in 90 per cent ethyl alcohol for 1 or more minutes. Air-dry.
7. Overstain with Löffler's methylene blue for 5 minutes.
8. Rinse gently in a large beaker of water. Drain off excess water.
9. Decolorize in alcohol until film is a pale blue color. Dry without blotting.
10. Adjust the microscope to give a field with a diameter of 0.205 mm. To do this use a 1.9 mm. (oil immersion) objective and a 6.4 × ocular; adjust the draw tube to give a field of the required size as measured by a micrometer slide.
11. Examine the milk film under the oil immersion lense of the adjusted microscope. Each field represents 1/300,000 ml. of milk. Count the bacteria in each of 30 different fields. Be sure to record fields containing no bacteria as zero.
12. Compute the number of bacteria per milliliter of milk by the following formula:  

$$\text{Average number of bacteria per field} \times 300,000 = \text{Number of bacteria per ml}$$

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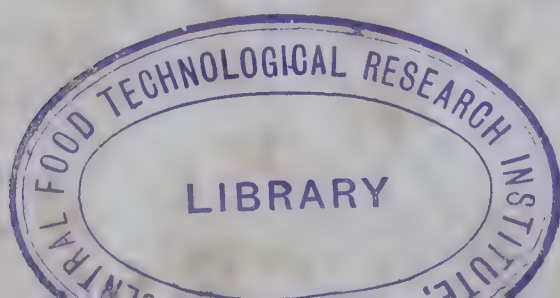


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